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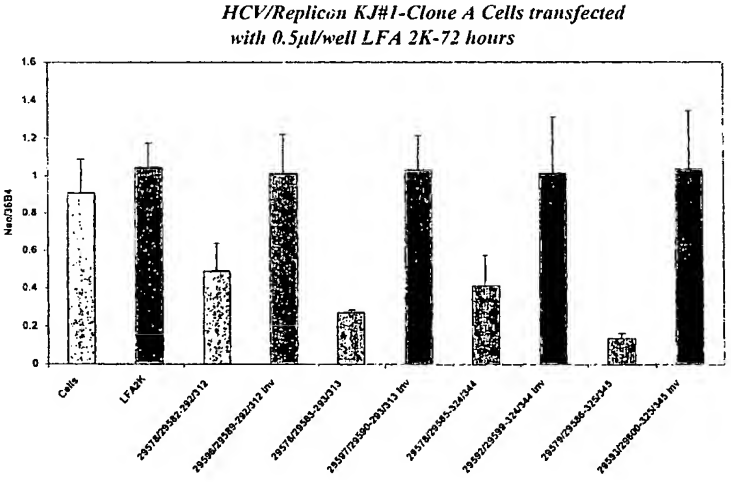
(54) Title: RNA INTERFERENCE MEDIATED INHIBITION OF GENE EXPRESSION USING CHEMICALLY MODIFIED SHORT INTERFERING NUCLEIC ACID



(57) Abstract: The present invention concerns methods and reagents useful in modulating gene expression in a variety of applications, including use in therapeutic, diagnostic, target validation, and genomic discovery applications. Specifically, the invention relates to synthetic chemically modified small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating RNA interference (RNAi) against target nucleic acid sequences. The small nucleic acid molecules are useful in the treatment of any disease or condition that responds to modulation of gene expression or activity in a cell, tissue, or organism.

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RNA INTERFERENCE MEDIATED INHIBITION OF GENE EXPRESSION USING CHEMICALLY MODIFIED SHORT INTERFERING NUCLEIC ACID (siNA)

This invention claims the benefit of Beigelman USSN 60/358,580 filed February 20, 2002, of Beigelman USSN 60/363,124 filed March 11, 2002, of Beigelman USSN 60/386,782 filed June 6, 2002, of Beigelman USSN 60/406,784 filed August 29,2002, of Beigelman USSN 60/408,378 filed September 5, 2002, of Beigelman USSN 60/409,293 filed September 9, 2002, and of Beigelman USSN 60/440,129 filed January 15, 2003. These applications are hereby incorporated by reference herein in their entireties, including the drawings.

Field Of The Invention

The present invention concerns methods and reagents useful in modulating gene expression in a variety of applications, including use in therapeutic, diagnostic, target validation, and genomic discovery applications. Specifically, the invention relates to synthetic small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of mediating RNA interference (RNAi).

Background Of The Invention

The following is a discussion of relevant art pertaining to RNAi. The discussion is provided only for understanding of the invention that follows. The summary is not an admission that any of the work described below is prior art to the claimed invention. Applicant demonstrates herein that chemically modified short interfering nucleic acids possess the same capacity to mediate RNAi as do siRNA molecules and are expected to possess improved stability and activity in vivo; therefore, this discussion is not meant to be limiting only to siRNA and can be applied to siNA as a whole.

RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Fire *et al.*, 1998, *Nature*, 391, 806). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in

fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla (Fire *et al.*, 1999, *Trends Genet.*, 15, 358). Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or from the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response though a mechanism that has yet to be fully characterized. This mechanism appears to be different from the interferon response that results from dsRNA-mediated activation of protein kinase PKR and 2',5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs) (Berstein *et al.*, 2001, *Nature*, 409, 363). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes (Elbashir *et al.*, 2001, *Genes Dev.*, 15, 188). Dicer has also been implicated in the excision of 21- and 22-nucleotide small temporal RNAs (stRNAs) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner *et al.*, 2001, *Science*, 293, 834). The RNAi response also features an endonuclease complex, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex (Elbashir *et al.*, 2001, *Genes Dev.*, 15, 188).

RNAi has been studied in a variety of systems. Fire *et al.*, 1998, *Nature*, 391, 806, were the first to observe RNAi in *C. elegans*. Wianny and Goetz, 1999, *Nature Cell Biol.*, 2, 70, describe RNAi mediated by dsRNA in mouse embryos. Hammond *et al.*, 2000, *Nature*, 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir *et al.*, 2001, *Nature*, 411, 494, describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human

embryonic kidney and HeLa cells. Recent work in Drosophila embryonic lysates (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877) has revealed certain requirements for siRNA length, structure, chemical composition, and sequence that are essential to mediate efficient RNAi activity. These studies have shown that 21-nucleotide siRNA duplexes are most active when containing 3'-terminal dinucleotide overhangs. Furthermore, complete substitution of one or both siRNA strands with 2'-deoxy (2'-H) or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of the 3'-terminal siRNA overhang nucleotides with 2'-deoxy nucleotides (2'-H) was shown to be tolerated. Single mismatch sequences in the center of the siRNA duplex were also shown to abolish RNAi activity. In addition, these studies also indicate that the position of the cleavage site in the target RNA is defined by the 5'-end of the siRNA guide sequence rather than the 3'-end of the guide sequence (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877). Other studies have indicated that a 5'-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA activity and that ATP is utilized to maintain the 5'-phosphate moiety on the siRNA (Nykanen *et al.*, 2001, *Cell*, 107, 309).

Studies have shown that replacing the 3'-terminal nucleotide overhanging segments of a 21-mer siRNA duplex having two -nucleotide 3'-overhangs with deoxyribonucleotides does not have an adverse effect on RNAi activity. Replacing up to four nucleotides on each end of the siRNA with deoxyribonucleotides has been reported to be well tolerated, whereas complete substitution with deoxyribonucleotides results in no RNAi activity (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877). In addition, Elbashir *et al.*, *supra*, also report that substitution of siRNA with 2'-O-methyl nucleotides completely abolishes RNAi activity. Li *et al.*, International PCT Publication No. WO 00/44914, and Beach *et al.*, International PCT Publication No. WO 01/68836 preliminarily suggest that siRNA may include modifications to either the phosphate-sugar backbone or the nucleoside to include at least one of a nitrogen or sulfur heteroatom, however, neither application postulates to what extent such modifications would be tolerated in siRNA molecules, nor provides any further guidance or examples of such modified siRNA. Kreutzer *et al.*, Canadian Patent Application No. 2,359,180, also describe certain chemical modifications for use in dsRNA constructs in order to counteract activation of double-stranded RNA-dependent protein kinase PKR, specifically 2'-amino or 2'-O-methyl nucleotides, and nucleotides containing a 2'-O or 4'-C methylene bridge.

However, Kreutzer *et al.* similarly fails to provide examples or guidance as to what extent these modifications would be tolerated in siRNA molecules.

Parrish *et al.*, 2000, *Molecular Cell*, 6, 1977-1087, tested certain chemical modifications targeting the unc-22 gene in *C. elegans* using long (>25 nt) siRNA transcripts. The authors describe the introduction of thiophosphate residues into these siRNA transcripts by incorporating thiophosphate nucleotide analogs with T7 and T3 RNA polymerase and observed that RNAs with two phosphorothioate modified bases also had substantial decreases in effectiveness as RNAi. Further, Parrish *et al.* reported that phosphorothioate modification of more than two residues greatly destabilized the RNAs *in vitro* such that interference activities could not be assayed. *Id.* at 1081. The authors also tested certain modifications at the 2'-position of the nucleotide sugar in the long siRNA transcripts and found that substituting deoxynucleotides for ribonucleotides produced a substantial decrease in interference activity, especially in the case of Uridine to Thymidine and/or Cytidine to deoxy-Cytidine substitutions. *Id.* In addition, the authors tested certain base modifications, including substituting, in sense and antisense strands of the siRNA, 4-thiouracil, 5-bromouracil, 5-iodouracil, and 3-(aminoallyl)uracil for uracil, and inosine for guanosine. Whereas 4-thiouracil and 5-bromouracil substitution appeared to be tolerated, Parrish reported that inosine produced a substantial decrease in interference activity when incorporated in either strand. Parrish also reported that incorporation of 5-iodouracil and 3-(aminoallyl)uracil in the antisense strand resulted in a substantial decrease in RNAi activity as well.

The use of longer dsRNA has been described. For example, Beach *et al.*, International PCT Publication No. WO 01/68836, describes specific methods for attenuating gene expression using endogenously-derived dsRNA. Tuschl *et al.*, International PCT Publication No. WO 01/75164, describe a *Drosophila in vitro* RNAi system and the use of specific siRNA molecules for certain functional genomic and certain therapeutic applications; although Tuschl, 2001, *Chem. Biochem.*, 2, 239-245, doubts that RNAi can be used to cure genetic diseases or viral infection due to the danger of activating interferon response. Li *et al.*, International PCT Publication No. WO 00/44914, describe the use of specific dsRNAs for attenuating the expression of certain target genes. Zernicka-Goetz *et al.*, International PCT Publication No. WO 01/36646, describe certain methods for inhibiting the expression of particular genes in mammalian

cells using certain dsRNA molecules. Fire *et al.*, International PCT Publication No. WO 99/32619, describe particular methods for introducing certain dsRNA molecules into cells for use in inhibiting gene expression. Plaetinck *et al.*, International PCT Publication No. WO 00/01846, describe certain methods for identifying specific genes responsible for conferring a particular phenotype in a cell using specific dsRNA molecules. Mello *et al.*, International PCT Publication No. WO 01/29058, describe the identification of specific genes involved in dsRNA-mediated RNAi. Deschamps Depaillette *et al.*, International PCT Publication No. WO 99/07409, describe specific compositions consisting of particular dsRNA molecules combined with certain anti-viral agents. Waterhouse *et al.*, International PCT Publication No. 99/53050, describe certain methods for decreasing the phenotypic expression of a nucleic acid in plant cells using certain dsRNAs. Driscoll *et al.*, International PCT Publication No. WO 01/49844, describe specific DNA constructs for use in facilitating gene silencing in targeted organisms.

Others have reported on various RNAi and gene-silencing systems. For example, Parrish *et al.*, 2000, *Molecular Cell*, 6, 1977-1087, describe specific chemically-modified siRNA constructs targeting the unc-22 gene of *C. elegans*. Grossniklaus, International PCT Publication No. WO 01/38551, describes certain methods for regulating polycomb gene expression in plants using certain dsRNAs. Churikov *et al.*, International PCT Publication No. WO 01/42443, describe certain methods for modifying genetic characteristics of an organism using certain dsRNAs. Cogoni *et al.*, International PCT Publication No. WO 01/53475, describe certain methods for isolating a Neurospora silencing gene and uses thereof. Reed *et al.*, International PCT Publication No. WO 01/68836, describe certain methods for gene silencing in plants. Honer *et al.*, International PCT Publication No. WO 01/70944, describe certain methods of drug screening using transgenic nematodes as Parkinson's Disease models using certain dsRNAs. Deak *et al.*, International PCT Publication No. WO 01/72774, describe certain *Drosophila*-derived gene products that may be related to RNAi .in *Drosophila*. Arndt *et al.*, International PCT Publication No. WO 01/92513 describe certain methods for mediating gene suppression by using factors that enhance RNAi. Tuschl *et al.*, International PCT Publication No. WO 02/44321, describe certain synthetic siRNA constructs. Pachuk *et al.*, International PCT Publication No. WO 00/63364, and Satishchandran *et al.*, International PCT Publication No. WO 01/04313, describe certain

methods and compositions for inhibiting the function of certain polynucleotide sequences using certain dsRNAs. Echeverri *et al.,* International PCT Publication No. WO 02/38805, describe certain *C. elegans* genes identified via RNAi. Kreutzer *et al.,* International PCT Publications Nos. WO 02/055692, WO 02/055693, and EP 1144623 B1 describes certain methods for inhibiting gene expression using RNAi. Graham *et al.,* International PCT Publications Nos. WO 99/49029 and WO 01/70949, and AU 4037501 describe certain vector expressed siRNA molecules. Fire *et al.,* US 6,506,559, describe certain methods for inhibiting gene expression in vitro using certain long dsRNA (greater than 25 nucleotide) constructs that mediate RNAi.

SUMMARY OF THE INVENTION

This invention relates to compounds, compositions, and methods useful for modulating RNA function and/or gene expression in a cell. Specifically, the instant invention features synthetic small nucleic acid molecules, such as short interfering nucleic acid (siNA), short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), and short hairpin RNA (shRNA) molecules capable of modulating gene expression in cells by RNA inference (RNAi). The siNA molecules of the invention can be chemically modified. The use of chemically modified siNA can improve various properties of native siRNA molecules through increased resistance to nuclease degradation *in vivo* and/or improved cellular uptake. The chemically modified siNA molecules of the instant invention provide useful reagents and methods for a variety of therapeutic, diagnostic, agricultural, target validation, genomic discovery, genetic engineering and pharmacogenomic applications.

In a non-limiting example, the introduction of chemically modified nucleotides into nucleic acid molecules provides a powerful tool in overcoming potential limitations of *in vivo* stability and bioavailability inherent to native RNA molecules that are delivered exogenously. For example, the use of chemically modified nucleic acid molecules can enable a lower dose of a particular nucleic acid molecule for a given therapeutic effect since chemically modified nucleic acid molecules tend to have a longer half-life in serum. Furthermore, certain chemical modifications can improve the bioavailability of nucleic acid molecules by targeting particular cells or tissues and/or improving cellular uptake of the nucleic acid molecule. Therefore, even if the activity of a chemically modified

nucleic acid molecule is reduced as compared to a native nucleic acid molecule, for example when compared to an all RNA nucleic acid molecule, the overall activity of the modified nucleic acid molecule can be greater than the native molecule due to improved stability and/or delivery of the molecule. Unlike native unmodified siRNA, chemically modified siNA can also minimize the possibility of activating interferon activity in humans.

In one embodiment, the nucleic acid molecules of the invention that act as mediators of the RNA interference gene silencing response are chemically modified double stranded nucleic acid molecules. As in their native double stranded RNA counterparts, these siNA molecules typically consist of duplexes containing about 19 base pairs between oligonucleotides comprising about 19 to about 25 nucleotides. The most active siRNA molecules are thought to have such duplexes with overhanging ends of 1-3 nucleotides, for example 21 nucleotide duplexes with 19 base pairs and 2 nucleotide 3'-overhangs. These overhanging segments are readily hydrolyzed by endonucleases *in vivo*. Studies have shown that replacing the 3'-overhanging segments of a 21-mer siRNA duplex having 2 nucleotide 3' overhangs with deoxyribonucleotides does not have an adverse effect on RNAi activity. Replacing up to 4 nucleotides on each end of the siRNA with deoxyribonucleotides has been reported to be well tolerated whereas complete substitution with deoxyribonucleotides results in no RNAi activity (Elbashir et al., 2001, EMBO J., 20, 6877). In addition, Elbashir *et al, supra*, also report that substitution of siRNA with 2'-O-methyl nucleotides completely abolishes RNAi activity. Li *et al.*, International PCT Publication No. WO 00/44914, and Beach *et al.*, International PCT Publication No. WO 01/68836 both suggest that siRNA may include modifications to either the phosphate-sugar back bone or the nucleoside to include at least one of a nitrogen or sulfur heteroatom, however neither application teaches to what extent these modifications are tolerated in siRNA molecules nor provide any examples of such modified siRNA. Kreutzer and Limmer, Canadian Patent Application No. 2,359,180, also describe certain chemical modifications for use in dsRNA constructs in order to counteract activation of double stranded-RNA-dependent protein kinase PKR, specifically 2'-amino or 2'-O-methyl nucleotides, and nucleotides containing a 2'-O or 4'-C methylene bridge. However, Kreutzer and Limmer similarly fail to show to what

extent these modifications are tolerated in siRNA molecules nor provide any examples of such modified siRNA.

In one embodiment, the invention features chemically modified siNA constructs having specificity for target nucleic acid molecules in a cell. Non-limiting examples of such chemical modifications include without limitation phosphorothioate internucleotide linkages, 2'-O-methyl ribonucleotides, 2'-deoxy-2'-fluoro ribonucleotides, 2'-deoxy ribonucleotides, "universal base" nucleotides, 5-C-methyl nucleotides, and inverted deoxyabasic residue incorporation. These chemical modifications, when used in various siNA constructs, are shown to preserve RNAi activity in cells while at the same time, dramatically increasing the serum stability of these compounds. Furthermore, contrary to the data published by Parrish *et al., supra,* applicant demonstrates that multiple (greater than one) phosphorothioate substitutions are well-tolerated and confer substantial increases in serum stability for modified siNA constructs.

In one embodiment, the chemically-modified siNA molecules of the invention comprise a duplex having two strands, one or both of which can be chemically-modified, wherein each strand is about 19 to about 29 (e.g., about 19, 20, 21, 22, 23, 24, 25, 26, 27, 28,or 29) nucleotides. In one embodiment, the chemically-modified siNA molecules of the invention comprise a duplex having two strands, one or both of which can be chemically-modified, wherein each strand is about 19 to about 23 (e.g., about 19, 20, 21, 22, or 23) nucleotides. In one embodiment, a siNA molecule of the invention comprises modified nucleotides while maintaining the ability to mediate RNAi. The modified nucleotides can be used to improve *in vitro* or *in vivo* characteristics such as stability, activity, and/or bioavailability. For example, a siNA molecule of the invention can comprise modified nucleotides as a percentage of the total number of nucleotides present in the siNA molecule. As such, a siNA molecule of the invention can generally comprise modified nucleotides from about 5 to about 100% of the nucleotide positions (e.g., 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 100% of the nucleotide positions). The actual percentage of modified nucleotides present in a given siNA molecule depends on the total number of nucleotides present in the siNA. If the siNA molecule is single stranded, the percent modification can be based upon the total number of nucleotides present in the single stranded siNA molecules. Likewise, if the siNA molecule is double stranded, the percent modification

can be based upon the total number of nucleotides present in the sense strand, antisense strand, or both the sense and antisense strands. In addition, the actual percentage of modified nucleotides present in a given siNA molecule can also depend on the total number of purine and pyrimidine nucleotides present in the siNA, for example, wherein all pyrimidine nucleotides and/or all purine nucleotides present in the siNA molecule are modified.

The antisense region of a siNA molecule of the invention can comprise a phosphorothioate internucleotide linkage at the 3'-end of said antisense region. The antisense region can comprise about one to about five phosphorothioate internucleotide linkages at the 5'-end of said antisense region. The 3'-terminal nucleotide overhangs of a siNA molecule of the invention can comprise ribonucleotides or deoxyribonucleotides that are chemically-modified at a nucleic acid sugar, base, or backbone. The 3'-terminal nucleotide overhangs can comprise one or more universal base ribonucleotides. The 3'-terminal nucleotide overhangs can comprise one or more acyclic nucleotides.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a target gene, wherein the siNA molecule comprises no ribonucleotides and each strand of the double-stranded siNA comprises about 21 nucleotides.

In one embodiment, one of the strands of a double-stranded siNA molecule of the invention comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of a target gene, and wherein the second strand of a double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or a portion thereof of the target gene.

In one embodiment, a siNA molecule of the invention comprises about 19 to about 23 nucleotides, and each strand comprises at least about 19 nucleotides that are complementary to the nucleotides of the other strand.

In one embodiment, a siNA molecule of the invention comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of a target gene, and the siNA further comprises a sense region, wherein the sense region comprises a nucleotide sequence substantially similar to the

nucleotide sequence or a portion thereof of the target gene. The antisense region and the sense region each comprise about 19 to about 23 nucleotides, and the antisense region comprises at least about 19 nucleotides that are complementary to nucleotides of the sense region.

In one embodiment, a siNA molecule of the invention comprises a sense region and an antisense region, wherein the antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of RNA encoded by a target gene and the sense region comprises a nucleotide sequence that is complementary to the antisense region.

In one embodiment, a siNA molecule of the invention is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of the siNA molecule. In another embodiment, the sense region is connected to the antisense region via a linker molecule, which can be a polynucleotide linker or a non-nucleotide linker.

In one embodiment, a siNA molecule of the invention comprises a sense region and antisense region, wherein pyrimidine nucleotides in the sense region compries 2'-O-methyl pyrimidine nucleotides and purine nucleotides in the sense region comprise 2'-deoxy purine nucleotides.

In one embodiment, a siNA molecule of the invention comprises a sense region and antisense region, wherein pyrimidine nucleotides present in the sense region comprise 2'-deoxy-2'-fluoro pyrimidine nucleotides and wherein purine nucleotides present in the sense region comprise 2'-deoxy purine nucleotides.

In one embodiment, a siNA molecule of the invention comprises a sense region and antisense region, wherein the sense region includes a terminal cap moiety at the 5'-end, the 3'-end, or both of the 5' and 3' ends of the sense region. In another embodiment, the terminal cap moiety is an inverted deoxy abasic moiety.

In one embodiment, a siNA molecule of the invention comprises a sense region and antisense region, wherein pyrimidine nucleotides of the antisense region comprise 2'-

deoxy-2'-fluoro pyrimidine nucleotides and purine nucleotides of the antisense region comprise 2'-O-methyl purine nucleotides.

In one embodiment, a siNA molecule of the invention comprises a sense region and antisense region, wherein pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and wherein purine nucleotides present in the antisense region comprise 2'-deoxy- purine nucleotides.

In one embodiment, a siNA molecule of the invention comprises a sense region and antisense region, wherein the antisense region comprises a phosphorothioate internucleotide linkage at the 3' end of the antisense region.

In one embodiment, a siNA molecule of the invention comprises a sense region and antisense region, wherein the antisense region comprises a glyceryl modification at the 3' end of the antisense region.

In one embodiment, a siNA molecule of the invention is assembled from two separate oligonucleotide fragments, wherein each of the two fragments of the siNA molecule comprise 21 nucleotides.

In one embodiment, a siNA molecule of the invention is assembled from two separate oligonucleotide fragments, wherein about 19 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule and wherein at least two 3' terminal nucleotides of each fragment of the siNA molecule are not base-paired to the nucleotides of the other fragment of the siNA molecule.

In one embodiment, a siNA molecule of the invention is assembled from two separate oligonucleotide fragments, wherein each of the two 3' terminal nucleotides of each fragment of the siNA molecule are 2'-deoxy-pyrimidines, such as 2'-deoxy-thymidine.

In one embodiment, a siNA molecule of the invention is assembled from two separate oligonucleotide fragments, wherein each of the two fragments of the siNA molecule comprise 21 nucleotides and wherein all 21 nucleotides of each fragment of the

siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule.

In one embodiment, a siNA molecule of the invention comprises a sense region and antisense region, wherein about 19 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by a target gene.

In one embodiment, a siNA molecule of the invention comprises a sense region and antisense region, wherein the siNA is assembled from two separate oligonucleotide fragments, wherein each of the two fragments of the siNA molecule comprise 21 nucleotides, and wherein 21 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by a target gene.

In one embodiment, a siNA molecule of the invention is assembled from two separate oligonucleotide fragments, wherein the 5'-end of a fragment comprising the antisense region of the siNA optionally includes a phosphate group.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a target RNA sequence, wherein the siNA molecule comprises no ribonucleotides and wherein each strand of the double-stranded siNA molecule comprises about 21 nucleotides.

In one embodiment, a target RNA sequence contemplated by the invention is encoded by a viral genome, bacterial gene, mammalian gene, human gene, or plant gene.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the replication of a virus (e.g, as mammalian virus, plant virus, hepatitis C virus, human immunodeficiency virus, hepatitis B virus, herpes simplex virus, cytomegalovirus, human papilloma virus, respiratory syncytial virus, or influenza virus) wherein the siNA molecule comprises no ribonucleotides and each strand of the double-stranded siNA molecule comprises about 21 nucleotides.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a target gene, wherein the siNA molecule does not require the presence of a ribonucleotide within the siNA

molecule for the inhibition of expression of a target gene and wherein each strand of the double-stranded siNA molecule comprises about 21 nucleotides .

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a target gene by mediating RNA interference (RNAi) process, wherein the siNA molecule comprises no ribonucleotides and wherein each strand of the double-stranded siNA molecule comprises about 21 nucleotides.

In one embodiment, the invention features a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the replication of a virus (e.g, as mammalian virus, plant virus, hepatitis C virus, human immunodeficiency virus, hepatitis B virus, herpes simplex virus, cytomegalovirus, human papilloma virus, respiratory syncytial virus, or influenza virus), wherein the siNA molecule does not require the presence of a ribonucleotide within the siNA molecule for the inhibition of replication of the virus and each strand of the double-stranded siNA molecule comprises about 21 nucleotides.

In one embodiment, the invention features a medicament comprising a siNA molecule of the invention.

In one embodiment, the invention features an active ingredient comprising a siNA molecule of the invention.

In another embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siNA) molecule to down-regulate expression of a target gene, wherein the siNA molecule comprises no ribonucleotides and each strand of the double-stranded siNA comprises about 21 nucleotides.

In another embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siNA) molecule to inhibit the expression of a target RNA sequence, wherein the siNA molecule comprises no ribonucleotides and wherein each strand of the double-stranded siNA molecule comprises about 21 nucleotides.

In another embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siNA) molecule to inhibit the replication of a virus, wherein the

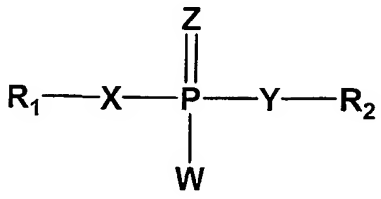
siNA molecule comprises no ribonucleotides and each strand of the double-stranded siNA molecule comprises about 21 nucleotides.

In another embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siNA) molecule to inhibit the expression of a target gene, wherein the siNA molecule does not require the presence of a ribonucleotide within the siNA molecule for the inhibition of expression of a target gene and wherein each strand of the double-stranded siNA molecule comprises about 21 nucleotides.

In another embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siNA) molecule to inhibit the expression of a target gene by mediating RNA interference (RNAi) process, wherein the siNA molecule comprises no ribonucleotides and wherein each strand of the double-stranded siNA molecule comprises about 21 nucleotides.

In another embodiment, the invention features the use of a double-stranded short interfering nucleic acid (siNA) molecule to inhibit the replication of a virus, wherein the siNA molecule does not require the presence of a ribonucleotide within the siNA molecule for the inhibition of replication of a virus and each strand of the double-stranded siNA molecule comprises about 21 nucleotides.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides comprising a backbone modified internucleotide linkage having Formula I:

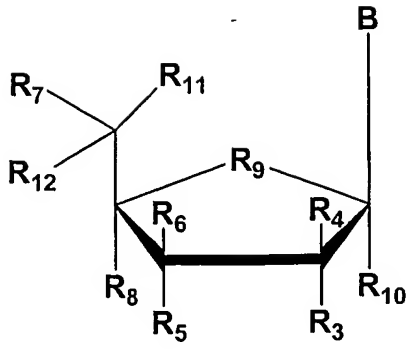


wherein each R1 and R2 is independently any nucleotide, non-nucleotide, or polynucleotide which can be naturally-occurring or chemically-modified, each X and Y is independently O, S, N, alkyl, or substituted alkyl, each Z and W is independently O, S, N,

alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, or aralkyl, and wherein W, X, Y, and Z are optionally not all O.

The chemically-modified internucleotide linkages having Formula I, for example, wherein any Z, W, X, and/or Y independently comprises a sulphur atom, can be present in one or both oligonucleotide strands of the siNA duplex, for example, in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) chemically-modified internucleotide linkages having Formula I at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified internucleotide linkages having Formula I at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) pyrimidine nucleotides with chemically-modified internucleotide linkages having Formula I in the sense strand, the antisense strand, or both strands. In yet another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) purine nucleotides with chemically-modified internucleotide linkages having Formula I in the sense strand, the antisense strand, or both strands. In another embodiment, a siNA molecule of the invention having internucleotide linkage(s) of Formula I also comprises a chemically-modified nucleotide or non-nucleotide having any of Formulae I-VII.

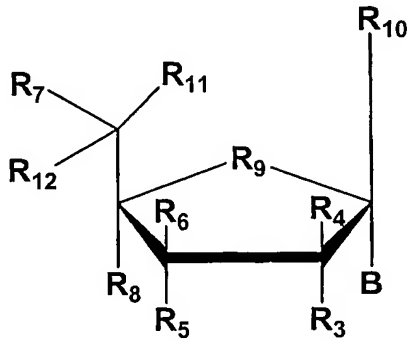
In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides or non-nucleotides having Formula II:



wherein each R3, R4, R5, R6, R7, R8, R10, R11 and R12 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or group having Formula I; R9 is O, S, CH2, S=O, CHF, or CF2, and B is a nucleosidic base such as adenine, guanine, uracil, cytosine, thymine, 2-aminoadenosine, 5-methylcytosine, 2,6-diaminopurine, or any other non-naturally occurring base that can be complementary or non-complementary to target RNA or a non-nucleosidic base such as phenyl, naphthyl, 3-nitropyrrole, 5-nitroindole, nebularine, pyridone, pyridinone, or any other non-naturally occurring universal base that can be complementary or non-complementary to target RNA.

The chemically-modified nucleotide or non-nucleotide of Formula II can be present in one or both oligonucleotide strands of the siNA duplex, for example in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more chemically-modified nucleotide or non-nucleotide of Formula II at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotides or non-nucleotides of Formula II at the 5'-end of the sense strand, the antisense strand, or both strands. In anther non-limiting example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotides or non-nucleotides of Formula II at the 3'-end of the sense strand, the antisense strand, or both strands.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) nucleotides or non-nucleotides having Formula III:



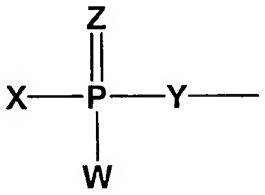
wherein each R3, R4, R5, R6, R7, R8, R10, R11 and R12 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or group having Formula I; R9 is O, S, CH2, S=O, CHF, or CF2, and B is a nucleosidic base such as adenine, guanine, uracil, cytosine, thymine, 2-aminoadenosine, 5-methylcytosine, 2,6-diaminopurine, or any other non-naturally occurring base that can be employed to be complementary or non-complementary to target RNA or a non-nucleosidic base such as phenyl, naphthyl, 3-nitropyrrole, 5-nitroindole, nebularine, pyridone, pyridinone, or any other non-naturally occurring universal base that can be complementary or non-complementary to target RNA.

The chemically-modified nucleotide or non-nucleotide of Formula III can be present in one or both oligonucleotide strands of the siNA duplex, for example, in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more chemically-modified nucleotide or non-nucleotide of Formula III at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-

modified nucleotide(s) or non-nucleotide(s) of Formula III at the 5'-end of the sense strand, the antisense strand, or both strands. In anther non-limiting example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) chemically-modified nucleotide or non-nucleotide of Formula III at the 3'-end of the sense strand, the antisense strand, or both strands.

In another embodiment, a siNA molecule of the invention comprises a nucleotide having Formula II or III, wherein the nucleotide having Formula II or III is in an inverted configuration. For example, the nucleotide having Formula II or III is connected to the siNA construct in a 3'-3', 3'-2', 2'-3', or 5'-5' configuration, such as at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both siNA strands.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises a 5'-terminal phosphate group having Formula IV:



wherein each X and Y is independently O, S, N, alkyl, substituted alkyl, or alkylhalo; wherein each Z and W is independently O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, aralkyl, or alkylhalo; and wherein W, X, Y and Z are not all O.

In one embodiment, the invention features a siNA molecule having a 5'-terminal phosphate group having Formula IV on the target-complementary strand, for example, a strand complementary to a target RNA, wherein the siNA molecule comprises an all RNA siNA molecule. In another embodiment, the invention features a siNA molecule having a 5'-terminal phosphate group having Formula IV on the target-complementary strand wherein the siNA molecule also comprises about 1 to about 3 (*e.g.*, about 1, 2, or 3) nucleotide 3'-terminal nucleotide overhangs having about 1 to about 4 (*e.g.*, about 1, 2, 3, or 4) deoxyribonucleotides on the 3'-end of one or both strands. In another embodiment, a 5'-terminal phosphate group having Formula IV is present on the target-complementary

strand of a siNA molecule of the invention, for example a siNA molecule having chemical modifications having any of Formulae I-VII.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises one or more phosphorothioate internucleotide linkages. For example, in a non-limiting example, the invention features a chemically-modified short interfering nucleic acid (siNA) having about 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages in one siNA strand. In yet another embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) individually having about 1, 2, 3, 4, 5, 6, 7, 8 or more phosphorothioate internucleotide linkages in both siNA strands. The phosphorothioate internucleotide linkages can be present in one or both oligonucleotide strands of the siNA duplex, for example in the sense strand, the antisense strand, or both strands. The siNA molecules of the invention can comprise one or more phosphorothioate internucleotide linkages at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand, the antisense strand, or both strands. For example, an exemplary siNA molecule of the invention can comprise about 1 to about 5 or more (*e.g.*, about 1, 2, 3, 4, 5, or more) consecutive phosphorothioate internucleotide linkages at the 5'-end of the sense strand, the antisense strand, or both strands. In another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) pyrimidine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands. In yet another non-limiting example, an exemplary siNA molecule of the invention can comprise one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) purine phosphorothioate internucleotide linkages in the sense strand, the antisense strand, or both strands.

In one embodiment, the invention features a siNA molecule, wherein the sense strand comprises one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or about one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 10 or

more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

In another embodiment, the invention features a siNA molecule, wherein the sense strand comprises about 1 to about 5, specifically about 1, 2, 3, 4, or 5 phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5, or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more, pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without about 1 to about 5 or more, for example about 1, 2, 3, 4, 5, or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

In one embodiment, the invention features a siNA molecule, wherein the antisense strand comprises one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more phosphorothioate internucleotide linkages, and/or about one or more (*e.g.*, about 1, 2, 3,

4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 10 or more, specifically about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without one or more, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more phosphorothioate internucleotide linkages and/or a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3' and 5'-ends, being present in the same or different strand.

In another embodiment, the invention features a siNA molecule, wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the sense strand; and wherein the antisense strand comprises about 1 to about 5 or more, specifically about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) 2'-deoxy, 2'-O-methyl, 2'-deoxy-2'-fluoro, and/or one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) universal base modified nucleotides, and optionally a terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of the antisense strand. In another embodiment, one or more, for example about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more pyrimidine nucleotides of the sense and/or antisense siNA strand are chemically-modified with 2'-deoxy, 2'-O-methyl and/or 2'-deoxy-2'-fluoro nucleotides, with or without about 1 to about 5, for example about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages and/or a

terminal cap molecule at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends, being present in the same or different strand.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule having about 1 to about 5, specifically about 1, 2, 3, 4, 5 or more phosphorothioate internucleotide linkages in each strand of the siNA molecule.

In another embodiment, the invention features a siNA molecule comprising 2'-5' internucleotide linkages. The 2'-5' internucleotide linkage(s) can be at the 3'-end, the 5'-end, or both of the 3'- and 5'-ends of one or both siNA sequence strands. In addition, the 2'-5' internucleotide linkage(s) can be present at various other positions within one or both siNA sequence strands, for example, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more including every internucleotide linkage of a pyrimidine nucleotide in one or both strands of the siNA molecule can comprise a 2'-5' internucleotide linkage, or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more including every internucleotide linkage of a purine nucleotide in one or both strands of the siNA molecule can comprise a 2'-5' internucleotide linkage.

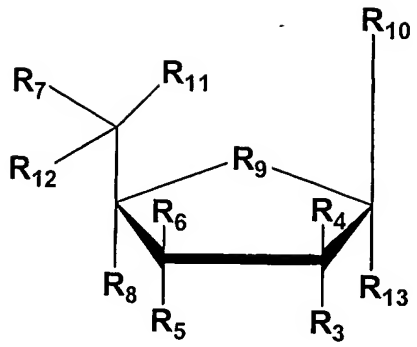
In another embodiment, a chemically-modified siNA molecule of the invention comprises a duplex having two strands, one or both of which can be chemically-modified, wherein each strand is about 18 to about 27 (*e.g.*, about 18, 19, 20, 21, 22, 23, 24, 25, 26, or 27) nucleotides in length, wherein the duplex has about 18 to about 23 (*e.g.*, about 18, 19, 20, 21, 22, or 23) base pairs, and wherein the chemical modification comprises a structure having any of Formulae I-VII. For example, an exemplary chemically-modified siNA molecule of the invention comprises a duplex having two strands, one or both of which can be chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein each strand consists of about 21 nucleotides, each having a 2-nucleotide 3'-terminal nucleotide overhang, and wherein the duplex has about 19 base pairs. In another embodiment, a siNA molecule of the invention comprises a single stranded hairpin structure, wherein the siNA is about 36 to about 70 (*e.g.*, about 36, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 18 to about 23 (*e.g.*, about 18, 19, 20, 21, 22, or 23) base pairs, and wherein the siNA can include a chemical modification comprising a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a linear oligonucleotide having about 42 to about 50 (*e.g.*, about 42, 43, 44, 45,

46, 47, 48, 49, or 50) nucleotides that is chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein the linear oligonucleotide forms a hairpin structure having about 19 base pairs and a 2-nucleotide 3'-terminal nucleotide overhang. In another embodiment, a linear hairpin siNA molecule of the invention contains a stem loop motif, wherein the loop portion of the siNA molecule is biodegradable. For example, a linear hairpin siNA molecule of the invention is designed such that degradation of the loop portion of the siNA molecule *in vivo* can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

In another embodiment, a siNA molecule of the invention comprises a circular nucleic acid molecule, wherein the siNA is about 38 to about 70 (*e.g.*, about 38, 40, 45, 50, 55, 60, 65, or 70) nucleotides in length having about 18 to about 23 (*e.g.*, about 18, 19, 20, 21, 22, or 23) base pairs, and wherein the siNA can include a chemical modification, which comprises a structure having any of Formulae I-VII or any combination thereof. For example, an exemplary chemically-modified siNA molecule of the invention comprises a circular oligonucleotide having about 42 to about 50 (*e.g.*, about 42, 43, 44, 45, 46, 47, 48, 49, or 50) nucleotides that is chemically-modified with a chemical modification having any of Formulae I-VII or any combination thereof, wherein the circular oligonucleotide forms a dumbbell shaped structure having about 19 base pairs and 2 loops.

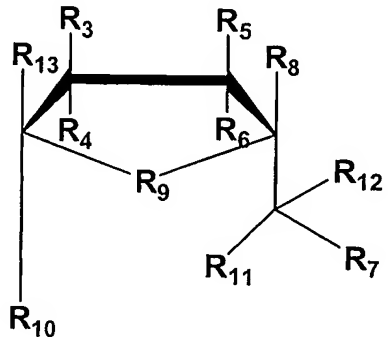
In another embodiment, a circular siNA molecule of the invention contains two loop motifs, wherein one or both loop portions of the siNA molecule is biodegradable. For example, a circular siNA molecule of the invention is designed such that degradation of the loop portions of the siNA molecule *in vivo* can generate a double-stranded siNA molecule with 3'-terminal overhangs, such as 3'-terminal nucleotide overhangs comprising about 2 nucleotides.

In one embodiment, a siNA molecule of the invention comprises at least one (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) abasic moiety, for example a compound having Formula V:



wherein each R3, R4, R5, R6, R7, R8, R10, R11, R12, and R13 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or group having Formula I; R9 is O, S, CH2, S=O, CHF, or CF2.

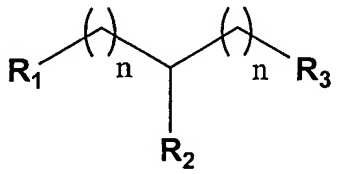
In one embodiment, a siNA molecule of the invention comprises at least one (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) inverted abasic moiety, for example a compound having Formula VI:



wherein each R3, R4, R5, R6, R7, R8, R10, R11, R12, and R13 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or group having Formula I; R9 is O, S, CH2, S=O, CHF, or CF2, and

either R2, R3, R8 or R13 serve as points of attachment to the siNA molecule of the invention.

In another embodiment, a siNA molecule of the invention comprises at least one (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) substituted polyalkyl moieties, for example a compound having Formula VII:



wherein each n is independently an integer from 1 to 12, each R1, R2 and R3 is independently H, OH, alkyl, substituted alkyl, alkaryl or aralkyl, F, Cl, Br, CN, CF3, OCF3, OCN, O-alkyl, S-alkyl, N-alkyl, O-alkenyl, S-alkenyl, N-alkenyl, SO-alkyl, alkyl-OSH, alkyl-OH, O-alkyl-OH, O-alkyl-SH, S-alkyl-OH, S-alkyl-SH, alkyl-S-alkyl, alkyl-O-alkyl, ONO2, NO2, N3, NH2, aminoalkyl, aminoacid, aminoacyl, ONH2, O-aminoalkyl, O-aminoacid, O-aminoacyl, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalklylamino, substituted silyl, or a group having Formula I, and R1, R2 or R3 serves as points of attachment to the siNA molecule of the invention.

In another embodiment, the invention features a compound having Formula VII, wherein R1 and R2 are hydroxyl (OH) groups, n = 1, and R3 comprises O and is the point of attachment to the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both strands of a double-stranded siNA molecule of the invention or to a single-stranded siNA molecule of the invention. This modification is referred to herein as "glyceryl" (for example modification 6 in **Figure 22**).

In another embodiment, a moiety having any of Formula V, VI or VII of the invention is at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of a siNA molecule of the invention. For example, a moiety having Formula V, VI or VII can be present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense strand, the sense strand, or both antisense and sense strands of the siNA molecule. In addition, a moiety having Formula VII can be present at the 3'-end or the 5'-end of a hairpin siNA molecule as described herein.

In another embodiment, a siNA molecule of the invention comprises an abasic residue having Formula V or VI, wherein the abasic residue having Formula VI or VI is connected to the siNA construct in a 3'-3', 3'-2', 2'-3', or 5'-5' configuration, such as at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of one or both siNA strands.

In one embodiment, a siNA molecule of the invention comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) locked nucleic acid (LNA) nucleotides, for example at the 5'-end, the 3'-end, both of the 5' and 3'-ends, or any combination thereof, of the siNA molecule.

In another embodiment, a siNA molecule of the invention comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) acyclic nucleotides, for example at the 5'-end, the 3'-end, both of the 5' and 3'-ends, or any combination thereof, of the siNA molecule.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises a sense region, where any (*e.g.*, one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where any (*e.g.*, one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides).

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises a sense region, where any (*e.g.*, one or more or all) pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where any (*e.g.*, one or more or all) purine nucleotides present in the sense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine

nucleotides), wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said sense region are 2'-deoxy nucleotides.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises an antisense region, where any (*e.g.*, one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (*e.g.*, one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides).

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises an antisense region, where any (*e.g.*, one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and wherein any (*e.g.*, one or more or all) purine nucleotides present in the antisense region are 2'-O-methyl purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides), wherein any nucleotides comprising a 3'-terminal nucleotide overhang that are present in said antisense region are 2'-deoxy nucleotides.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention, wherein the chemically-modified siNA comprises an antisense region, where any (*e.g.*, one or more or all) pyrimidine nucleotides present in the antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and where any (*e.g.*, one or more or all) purine nucleotides present in the antisense region are 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are

2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides).

In one embodiment, the invention features a chemically-modified short interfering nucleic acid (siNA) molecule of the invention capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system comprising a sense region and an antisense region. In one embodiment, the sense region comprises n one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more 2'-deoxy purine nucleotides (*e.g.*, wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides). The sense region can comprise inverted deoxy abasic modifications that are optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense region. The sense region can optionally further comprise a 3'-terminal overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxyribonucleotides. The antisense region comprisesone or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides). The antisense region can comprise a terminal cap modification, such as any modification described herein or shown in **Figure 22**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence. The antisense region optionally further comprises a 3'-terminal nucleotide overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides, wherein the overhang nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages. Non-limiting examples of these chemically-modified siNAs are shown in **Figures 18 and 19** and **Table IV** herein.

In another embodiment of the chemically-modified short interfering nucleic acid comprising a sense region and an antisense region, the sense region comprisesone or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more purine

ribonucleotides (e.g., wherein all purine nucleotides are purine ribonucleotides or alternately a plurality of purine nucleotides are purine ribonucleotides). The sense region can also compriseinverted deoxy abasic modifications that are optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense region. The sense region optionally further comprises a 3'-terminal overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxyribonucleotides. The antisense regioncomprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides). The antisense region can also comprise a terminal cap modification, such as any modification described herein or shown in **Figure 22**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence. The antisense region optionally further comprises a 3'-terminal nucleotide overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides, wherein the overhang nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages. Non-limiting examples of these chemically-modified siNAs are shown in **Figures 18 and 19** and **Table IV** herein.

In another embodiment of the chemically-modified short interfering nucleic acid comprising a sense region and an antisense region, the sense region comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (*e.g.*, wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more purine nucleotides selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides (*e.g.*, wherein all purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides or alternately a plurality of purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides). The sense region can comprise inverted

deoxy abasic modifications that are optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the sense region. The sense region can optionally further comprise a 3'-terminal overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxyribonucleotides. The antisense region comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more purine nucleotides selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides (e.g., wherein all purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides or alternately a plurality of purine nucleotides are selected from the group consisting of 2'-deoxy nucleotides, locked nucleic acid (LNA) nucleotides, 2'-methoxyethyl nucleotides, 4'-thionucleotides, and 2'-O-methyl nucleotides). The antisense can also comprisea terminal cap modification, such as any modification described herein or shown in **Figure 22**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence. The antisense region optionally further comprises a 3'-terminal nucleotide overhang having about 1 to about 4 (e.g., about 1, 2, 3, or 4) 2'-deoxynucleotides, wherein the overhang nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages.

In another embodiment, any modified nucleotides present in the siNA molecules of the invention, preferably in the antisense strand of the siNA molecules of the invention, but also optionally in the sense and/or both antisense and sense strands, comprise modified nucleotides having properties or characteristics similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag ed., 1984). As such, chemically modified nucleotides present in the siNA molecules of the invention, preferably in the antisense strand of the siNA molecules of the invention, but also optionally in the sense and/or both antisense and sense strands, are resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi. Non-

limiting examples of nucleotides having a northern configuration include locked nucleic acid (LNA) nucleotides (e.g., 2′-O,4′-C-methylene-(D-ribofuranosyl) nucleotides); 2'-methoxyethoxy (MOE) nucleotides; 2'-methyl-thio-ethyl, 2'-deoxy-2'-fluoro nucleotides, 2'-deoxy-2'-chloro nucleotides, 2'-azido nucleotides, and 2'-O-methyl nucleotides.

In one embodiment, the invention features a chemically-modified short interfering nucleic acid molecule (siNA) capable of mediating RNA interference (RNAi) inside a cell or reconstituted *in vitro* system, wherein the chemical modification comprises a conjugate attached to the chemically-modified siNA molecule. The conjugate can be attached to the chemically-modified siNA molecule via a covalent attachment. In one embodiment, the conjugate is attached to the chemically-modified siNA molecule via a biodegradable linker. In one embodiment, the conjugate molecule is attached at the 3'-end ofeither the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In another embodiment, the conjugate molecule is attached at the 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule. In yet another embodiment, the conjugate molecule is attached both the 3'-end and 5'-end of either the sense strand, the antisense strand, or both strands of the chemically-modified siNA molecule, or any combination thereof. In one embodiment, the conjugate molecule of the invention comprises a molecule that facilitates delivery of a chemically-modified siNA molecule into a biological system, such as a cell. In another embodiment, the conjugate molecule attached to the chemically-modified siNA molecule is a poly ethylene glycol, human serum albumin, or a ligand for a cellular receptor that can mediate cellular uptake. Examples of specific conjugate molecules contemplated by the instant invention that can be attached to chemically-modified siNA molecules are described in Vargeese *et al.,* U.S. Serial No. 10/201,394, incorporated by reference herein. The type of conjugates used and the extent of conjugation of siNA molecules of the invention can be evaluated for improved pharmacokinetic profiles, bioavailability, and/or stability of siNA constructs while at the same time maintaining the ability of the siNA to mediate RNAi activity. As such, one skilled in the art can screen siNA constructs that are modified with various conjugates to determine whether the siNA conjugate complex possesses improved properties while maintaining the ability to mediate RNAi, for example in animal models as are generally known in the art.

In one embodiment, the invention features a short interfering nucleic acid (siNA) molecule of the invention, wherein the siNA further comprises a nucleotide, non-nucleotide, or mixed nucleotide/non-nucleotide linker that joins the sense region of the siNA to the antisense region of the siNA. In one embodiment, a nucleotide linker of the invention can be a linker of ≥ 2 nucleotides in length, for example 3, 4, 5, 6, 7, 8, 9, or 10 nucleotides in length. In another embodiment, the nucleotide linker can be a nucleic acid aptamer. By "aptamer" or "nucleic acid aptamer" as used herein is meant a nucleic acid molecule that binds specifically to a target molecule wherein the nucleic acid molecule has sequence that comprises a sequence recognized by the target molecule in its natural setting. Alternately, an aptamer can be a nucleic acid molecule that binds to a target molecule where the target molecule does not naturally bind to a nucleic acid. The target molecule can be any molecule of interest. For example, the aptamer can be used to bind to a ligand-binding domain of a protein, thereby preventing interaction of the naturally occurring ligand with the protein. This is a non-limiting example and those in the art will recognize that other embodiments can be readily generated using techniques generally known in the art. (See, for example, Gold *et al.*, 1995, *Annu. Rev. Biochem.*, 64, 763; Brody and Gold, 2000, *J. Biotechnol.*, 74, 5; Sun, 2000, *Curr. Opin. Mol. Ther.*, 2, 100; Kusser, 2000, *J. Biotechnol.*, 74, 27; Hermann and Patel, 2000, *Science,* 287, 820; and Jayasena, 1999, *Clinical Chemistry,* 45, 1628.)

A non-nucleotide linker of the invention can comprise abasic nucleotide, polyether, polyamine, polyamide, peptide, carbohydrate, lipid, polyhydrocarbon, or other polymeric compounds (e.g. polyethylene glycols such as those having between 2 and 100 ethylene glycol units). Specific examples include those described by Seela and Kaiser, *Nucleic Acids Res.* 1990, *18*:6353 and *Nucleic Acids Res.* 1987, *15*:3113; Cload and Schepartz, *J. Am. Chem. Soc.* 1991, *113*:6324; Richardson and Schepartz, *J. Am. Chem. Soc.* 1991, *113*:5109; Ma *et al., Nucleic Acids Res.* 1993, *21*:2585 and *Biochemistry* 1993, *32*:1751; Durand *et al., Nucleic Acids Res.* 1990, *18*:6353; McCurdy *et al., Nucleosides & Nucleotides* 1991, *10*:287; Jschke *et al., Tetrahedron Lett.* 1993, *34*:301; Ono *et al., Biochemistry* 1991, *30*:9914; Arnold *et al.,* International Publication No. WO 89/02439; Usman *et al.,* International Publication No. WO 95/06731; Dudycz *et al.,* International Publication No. WO 95/11910 and Ferentz and Verdine, *J. Am. Chem. Soc.* 1991, *113*:4000, all hereby incorporated by reference herein. A "non-nucleotide" further means

any group or compound that can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound can be abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine, for example at the C1 position of the sugar.

In one embodiment, the invention features a short interfering nucleic acid (siNA) molecule capable of mediating RNA interference (RNAi) inside a cell or reconstituted in vitro system, wherein one or both strands of the siNA molecule that are assembled from two separate oligonucleotides do not comprise any ribonucleotides. In one embodiment, a siNA molecule can be assembled from a single oligonculeotide where the sense and antisense regions of the siNA comprise separate oligonucleotides not having any ribonucleotides (e.g., nucleotides having a 2'-OH group) present in the oligonucleotides. In another embodiment, a siNA molecule can be assembled from a single oligonculeotide where the sense and antisense regions of the siNA are linked or circularized by a nucleotide or non-nucleotide linker as desrcibed herein, wherein the oligonucleotide does not have any ribonucleotides (e.g., nucleotides having a 2'-OH group) present in the oligonucleotide. Applicant has surprisingly found that the presense of ribonucleotides (e.g., nucleotides having a 2'-hydroxyl group) within the siNA molecule is not required or essential to support RNAi activity. As such, in one embodiment, all positions within the siNA can include chemically modified nucleotides and/or non-nucleotides, such as nucleotides and or non-nucleotides having Formula I, II, III, IV, V, VI, or VII or any combination thereof to the extent that the ability of the siNA molecule to support RNAi activity in a cell is maintained.

In one embodiment, a siNA molecule of the invention is a single stranded siNA molecule that mediates RNAi activity in a cell or reconstituted in vitro system, wherein the siNA molecule comprises a single stranded polynucleotide having complementarity to a target nucleic acid sequence. The single stranded siNA molecule of the invention can comprise about 19 to about 29 nucleotides. In one embodiment, the single stranded siNA molecule of the invention comprises a 5'-terminal phosphate group. In another embodiment, the single stranded siNA molecule of the invention comprises a 5'-terminal phosphate group and a 3'-terminal phosphate group (e.g., a 2', 3'-cyclic phosphate). In

yet another embodiment, the single stranded siNA molecule of the invention comprises one or more chemically modified nucleotides or non-nucleotides described herein. For example, all the positions within the siNA molecule can include chemically-modified nucleotides such as nucleotides having any of Formulae I-VII, or any combination thereof to the extent that the ability of the siNA molecule to support RNAi activity in a cell is maintained.

In one embodiment, the single stranded siNA molecule having complementarity to a target nucleic acid sequence comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more 2'-O-methyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-O-methyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-O-methyl purine nucleotides.In another embodiment, the single stranded siNA molecule comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more 2'-deoxy purine nucleotides (e.g., wherein all purine nucleotides are 2'-deoxy purine nucleotides or alternately a plurality of purine nucleotides are 2'-deoxy purine nucleotides). In another embodiment, the single stranded siNA molecule comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), wherein any purine nucleotides present in the antisense region are locked nucleic acid (LNA) nucleotides (e.g., wherein all purine nucleotides are LNA nucleotides or alternately a plurality of purine nucleotides are LNA nucleotides). In another embodiment, the single stranded siNA molecule comprises one or more 2'-deoxy-2'-fluoro pyrimidine nucleotides (e.g., wherein all pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides or alternately a plurality of pyrimidine nucleotides are 2'-deoxy-2'-fluoro pyrimidine nucleotides), and one or more 2'-methoxyethyl purine nucleotides (e.g., wherein all purine nucleotides are 2'-methoxyethyl purine nucleotides or alternately a plurality of purine nucleotides are 2'-methoxyethyl purine nucleotides), the single stranded siNA can comprise a terminal cap modification, such as any

modification described herein or shown in **Figure 22**, that is optionally present at the 3'-end, the 5'-end, or both of the 3' and 5'-ends of the antisense sequence. The single stranded siNA optionally further comprises about 1 to about 4 (e.g., about 1, 2, 3, or 4) terminal 2'-deoxynucleotides at the 3'-end of the siNA molecule, wherein the terminal nucleotides can further comprise one or more (e.g., 1, 2, 3, or 4) phosphorothioate internucleotide linkages. The single stranded siNA optionally further comprises a terminal phosphate group, such as a 5'-terminal phosphate group.

In another embodiment, any modified nucleotides present in the single stranded siNA molecules of the invention comprise modified nucleotides having properties or characteristics similar to naturally occurring ribonucleotides. For example, the invention features siNA molecules including modified nucleotides having a Northern conformation (e.g., Northern pseudorotation cycle, see for example Saenger, *Principles of Nucleic Acid Structure*, Springer-Verlag ed., 1984). As such, chemically modified nucleotides present in the single stranded siNA molecules of the invention are preferably resistant to nuclease degradation while at the same time maintaining the capacity to mediate RNAi.

In one embodiment, the invention features a method for modulating the expression of a gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the gene; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the gene in the cell.

In one embodiment, the invention features a method for modulating the expression of a gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the gene and wherein the sense strand sequence of the siNA comprises a sequence substantially similar to the sequence of the target RNA; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the gene in the cell.

In another embodiment, the invention features a method for modulating the expression of more than one gene within a cell comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the genes; and (b) introducing

the siNA molecules into a cell under conditions suitable to modulate the expression of the genes in the cell.

In another embodiment, the invention features a method for modulating the expression of more than one gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the gene and wherein the sense strand sequence of the siNA comprises a sequence substantially similar to the sequence of the target RNA; and (b) introducing the siNA molecules into a cell under conditions suitable to modulate the expression of the genes in the cell.

In one embodiment, siNA molecules of the invention are used as reagents in ex vivo applications. For example, siNA reagents are intoduced into tissue or cells that are transplanted into a subject for therapeutic effect. The cells and/or tissue can be derived from an organism or subject that later receives the explant, or can be derived from another organism or subject prior to transplantation. The siNA molecules can be used to modulate the expression of one or more genes in the cells or tissue, such that the cells or tissue obtain a desired phenotype or are able to perform a function when transplanted in vivo. In one embodiment, certain target cells from a patient are extracted. These extracted cells are contacted with siNAs targeteing a specific nucleotide sequence within the cells under conditions suitable for uptake of the siNAs by these cells (e.g. using delivery reagents such as cationic lipids, liposomes and the like or using techniques such as electroporation to facilitate the delivery of siNAs into cells). The cells are then reintroduced back into the same patient or other patients. Non-limiting examples of ex vivo applications include use in organ/tissue transplant, tissue grafting, or treatment of pulmonary disease (e.g., restenosis) or prevent neointimal hyperplasia and atherosclerosis in vein grafts. Such ex vivo applications may also used to treat conditions associated with coronary and peripheral bypass graft failure, for example, such methods can be used in conjunction with peripheral vascular bypass graft surgery and coronary artery bypass graft surgery. Additional applications include transplants to treat CNS lesions or injury, including use in treatment of neurodegenerative conditions such as Alzheimer's disease, Parkinson's Disease, Epilepsy, Dementia, Huntington's disease, or amyotrophic lateral sclerosis (ALS).

In one embodiment, the invention features a method of modulating the expression of a gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the gene; and (b) introducing the siNA molecule into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the gene in that organism.

In one embodiment, the invention features a method of modulating the expression of a gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the gene and wherein the sense strand sequence of the siNA comprises a sequence substantially similar to the sequence of the target RNA; and (b) introducing the siNA molecule into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the gene in that organism.

In another embodiment, the invention features a method of modulating the expression of more than one gene in a tissue explant comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the genes; and (b) introducing the siNA molecules into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the genes in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the genes in that organism.

In one embodiment, the invention features a method of modulating the expression of a gene in an organism comprising: (a) synthesizing a siNA molecule of the invention,

which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the gene; and (b) introducing the siNA molecule into the organism under conditions suitable to modulate the expression of the gene in the organism.

In another embodiment, the invention features a method of modulating the expression of more than one gene in an organism comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein one of the siNA strands comprises a sequence complementary to RNA of the genes; and (b) introducing the siNA molecules into the organism under conditions suitable to modulate the expression of the genes in the organism.

In one embodiment, the invention features a method for modulating the expression of a gene within a cell comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the gene; and (b) introducing the siNA molecule into a cell under conditions suitable to modulate the expression of the gene in the cell.

In another embodiment, the invention features a method for modulating the expression of more than one gene within a cell comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the gene; and (b) contacting the siNA molecule with a cell in vitro or in vivo under conditions suitable to modulate the expression of the genes in the cell.

In one embodiment, the invention features a method of modulating the expression of a gene in a tissue explant comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the gene; and (b) contacting the siNA molecule with a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the gene in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the gene in that organism.

In another embodiment, the invention features a method of modulating the expression of more than one gene in a tissue explant comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the gene; and (b) introducing the siNA molecules into a cell of the tissue explant derived from a particular organism under conditions suitable to modulate the expression of the genes in the tissue explant. In another embodiment, the method further comprises introducing the tissue explant back into the organism the tissue was derived from or into another organism under conditions suitable to modulate the expression of the genes in that organism.

In one embodiment, the invention features a method of modulating the expression of a gene in an organism comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the gene; and (b) introducing the siNA molecule into the organism under conditions suitable to modulate the expression of the gene in the organism.

In another embodiment, the invention features a method of modulating the expression of more than one gene in an organism comprising: (a) synthesizing siNA molecules of the invention, which can be chemically-modified, wherein the siNA comprises a single stranded sequence having complementarity to RNA of the gene; and (b) introducing the siNA molecules into the organism under conditions suitable to modulate the expression of the genes in the organism.

In one embodiment, the invention features a method of modulating the expression of a gene in an organism comprising contacting the organism with a siNA molecule of the invention under conditions suitable to modulate the expression of the gene in the organism.

In another embodiment, the invention features a method of modulating the expression of more than one gene in an organism comprising contacting the organism with one or more siNA molecules of the invention under conditions suitable to modulate the expression of the genes in the organism.

The siNA molecules of the invention can be designed to inhibit target gene expression through RNAi targeting of a variety of RNA molecules. In one embodiment, the siNA molecules of the invention are used to target various RNAs corresponding to a target gene. Non-limiting examples of such RNAs include messenger RNA (mRNA), alternate RNA splice variants of target gene(s), post-transcriptionally modified RNA of target gene(s), pre-mRNA of target gene(s), and/or RNA templates. If alternate splicing produces a family of transcripts that are distinguished by usage of appropriate exons, the instant invention can be used to inhibit gene expression through the appropriate exons to specifically inhibit or to distinguish among the functions of gene family members. For example, a protein that contains an alternatively spliced transmembrane domain can be expressed in both membrane bound and secreted forms. Use of the invention to target the exon containing the transmembrane domain can be used to determine the functional consequences of pharmaceutical targeting of membrane bound as opposed to the secreted form of the protein. Non-limiting examples of applications of the invention relating to targeting these RNA molecules include therapeutic pharmaceutical applications, pharmaceutical discovery applications, molecular diagnostic and gene function applications, and gene mapping, for example using single nucleotide polymorphism mapping with siNA molecules of the invention. Such applications can be implemented using known gene sequences or from partial sequences available from an expressed sequence tag (EST).

In another embodiment, the siNA molecules of the invention are used to target conserved sequences corresponding to a gene family or gene families. As such, siNA molecules targeting multiple gene targets can provide increased therapeutic effect. In addition, siNA can be used to characterize pathways of gene function in a variety of applications. For example, the present invention can be used to inhibit the activity of target gene(s) in a pathway to determine the function of uncharacterized gene(s) in gene function analysis, mRNA function analysis, or translational analysis. The invention can be used to determine potential target gene pathways involved in various diseases and conditions toward pharmaceutical development. The invention can be used to understand pathways of gene expression involved in, for example, in development, such as prenatal development and postnatal development, and/or the progression and/or maintenance of cancer, infectious disease, autoimmunity, inflammation, endocrine disorders, renal

disease, pulmonary disease, cardiovascular disease, birth defects, ageing, any other disease or condition related to gene expression.

In one embodiment, the invention features a method comprising: (a) generating a library of siNA constructs having a predetermined complexity; and (b) assaying the siNA constructs of (a) above, under conditions suitable to determine RNAi target sites within the target RNA sequence. In another embodiment, the siNA molecules of (a) have strands of a fixed length, for example, about 23 nucleotides in length. In yet another embodiment, the siNA molecules of (a) are of differing length, for example having strands of about 19 to about 25 (*e.g.*, about 19, 20, 21, 22, 23, 24, or 25) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. In another embodiment, fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNAse protection assays, to determine the most suitable target site(s) within the target RNA sequence. The target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by cellular expression in *in vivo* systems.

In one embodiment, the invention features a method comprising: (a) generating a randomized library of siNA constructs having a predetermined complexity, such as of 4^N, where N represents the number of base paired nucleotides in each of the siNA construct strands (*eg.* for a siNA construct having 21 nucleotide sense and antisense strands with 19 base pairs, the complexity would be 4^{19}); and (b) assaying the siNA constructs of (a) above, under conditions suitable to determine RNAi target sites within the target RNA sequence. In another embodiment, the siNA molecules of (a) have strands of a fixed length, for example about 23 nucleotides in length. In yet another embodiment, the siNA molecules of (a) are of differing length, for example having strands of about 19 to about 25 (*e.g.*, about 19, 20, 21, 22, 23, 24, or 25) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described in Example 7 herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. In another embodiment, fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNAse protection assays, to determine the most suitable target site(s) within

the target RNA sequence. In another embodiment, the target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by cellular expression in *in vivo* systems.

In another embodiment, the invention features a method comprising: (a) analyzing the sequence of a RNA target encoded by a target gene; (b) synthesizing one or more sets of siNA molecules having sequence complementary to one or more regions of the RNA of (a); and (c) assaying the siNA molecules of (b) under conditions suitable to determine RNAi targets within the target RNA sequence. In one embodiment, the siNA molecules of (b) have strands of a fixed length, for example about 23 nucleotides in length. In another embodiment, the siNA molecules of (b) are of differing length, for example having strands of about 19 to about 25 (*e.g.*, about 19, 20, 21, 22, 23, 24, or 25) nucleotides in length. In one embodiment, the assay can comprise a reconstituted *in vitro* siNA assay as described herein. In another embodiment, the assay can comprise a cell culture system in which target RNA is expressed. Fragments of target RNA are analyzed for detectable levels of cleavage, for example by gel electrophoresis, northern blot analysis, or RNAse protection assays, to determine the most suitable target site(s) within the target RNA sequence. The target RNA sequence can be obtained as is known in the art, for example, by cloning and/or transcription for *in vitro* systems, and by expression in *in vivo* systems.

By "target site" is meant a sequence within a target RNA that is "targeted" for cleavage mediated by a siNA construct which contains sequences within its antisense region that are complementary to the target sequence.

By "detectable level of cleavage" is meant cleavage of target RNA (and formation of cleaved product RNAs) to an extent sufficient to discern cleavage products above the background of RNAs produced by random degradation of the target RNA. Production of cleavage products from 1-5% of the target RNA is sufficient to detect above the background for most methods of detection.

In one embodiment, the invention features a composition comprising a siNA molecule of the invention, which can be chemically-modified, in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a pharmaceutical composition comprising siNA molecules of the invention, which can be

chemically-modified, targeting one or more genes in a pharmaceutically acceptable carrier or diluent. In another embodiment, the invention features a method for treating or preventing a disease or condition in a subject comprising administering to the subject a composition of the invention under conditions suitable for the treatment or prevention of the disease or condition in the subject, alone or in conjunction with one or more other therapeutic compounds. In yet another embodiment, the invention features a method for reducing or preventing tissue rejection in a subject comprising administering to the subject a composition of the invention under conditions suitable for the reduction or prevention of tissue rejection in the subject.

In another embodiment, the invention features a method for validating a gene target, comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of a target gene; (b) introducing the siNA molecule into a cell, tissue, or organism under conditions suitable for modulating expression of the target gene in the cell, tissue, or organism; and (c) determining the function of the gene by assaying for any phenotypic change in the cell, tissue, or organism.

In another embodiment, the invention features a method for validating a target gene comprising: (a) synthesizing a siNA molecule of the invention, which can be chemically-modified, wherein one of the siNA strands includes a sequence complementary to RNA of a target gene; (b) introducing the siNA molecule into a biological system under conditions suitable for modulating expression of the target gene in the biological system; and (c) determining the function of the gene by assaying for any phenotypic change in the biological system.

By "biological system" is meant, material, in a purified or unpurified form, from biological sources, including but not limited to human, animal, plant, insect, bacterial, viral or other sources, wherein the system comprises the components required for RNAi acitivity. The term "biological system" includes, for example, a cell, tissue, or organism, or extract thereof. The term biological system also includes reconstituted RNAi systems that can be used in an *in vitro* setting.

By "phenotypic change" is meant any detectable change to a cell that occurs in response to contact or treatment with a nucleic acid molecule of the invention (e.g.,

siNA). Such detectable changes include, but are not limited to, changes in shape, size, proliferation, motility, protein expression or RNA expression or other physical or chemical changes as can be assayed by methods known in the art. The detectable change can also include expression of reporter genes/molecules such as Green Florescent Protein (GFP) or various tags that are used to identify an expressed protein or any other cellular component that can be assayed.

In one embodiment, the invention features a kit containing a siNA molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of a target gene in a cell, tissue, or organism. In another embodiment, the invention features a kit containing more than one siNA molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of more than one target gene in a cell, tissue, or organism.

In one embodiment, the invention features a kit containing a siNA molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of a target gene in a biological system. In another embodiment, the invention features a kit containing more than one siNA molecule of the invention, which can be chemically-modified, that can be used to modulate the expression of more than one target gene in a biological system.

In one embodiment, the invention features a cell containing one or more siNA molecules of the invention, which can be chemically-modified. In another embodiment, the cell containing a siNA molecule of the invention is a mammalian cell. In yet another embodiment, the cell containing a siNA molecule of the invention is a human cell.

In one embodiment, the synthesis of a siNA molecule of the invention, which can be chemically-modified, comprises: (a) synthesis of two complementary strands of the siNA molecule; (b) annealing the two complementary strands together under conditions suitable to obtain a double-stranded siNA molecule. In another embodiment, synthesis of the two complementary strands of the siNA molecule is by solid phase oligonucleotide synthesis. In yet another embodiment, synthesis of the two complementary strands of the siNA molecule is by solid phase tandem oligonucleotide synthesis.

In one embodiment, the invention features a method for synthesizing a siNA duplex molecule comprising: (a) synthesizing a first oligonucleotide sequence strand of the siNA molecule, wherein the first oligonucleotide sequence strand comprises a cleavable linker molecule that can be used as a scaffold for the synthesis of the second oligonucleotide sequence strand of the siNA; (b) synthesizing the second oligonucleotide sequence strand of siNA on the scaffold of the first oligonucleotide sequence strand, wherein the second oligonucleotide sequence strand further comprises a chemical moiety than can be used to purify the siNA duplex; (c) cleaving the linker molecule of (a) under conditions suitable for the two siNA oligonucleotide strands to hybridize and form a stable duplex; and (d) purifying the siNA duplex utilizing the chemical moiety of the second oligonucleotide sequence strand. In one embodiment, cleavage of the linker molecule in (c) above takes place during deprotection of the oligonucleotide, for example under hydrolysis conditions using an alkylamine base such as methylamine. In one embodiment, the method of synthesis comprises solid phase synthesis on a solid support such as controlled pore glass (CPG) or polystyrene, wherein the first sequence of (a) is synthesized on a cleavable linker, such as a succinyl linker, using the solid support as a scaffold. The cleavable linker in (a) used as a scaffold for synthesizing the second strand can comprise similar reactivity as the solid support derivatized linker, such that cleavage of the solid support derivatized linker and the cleavable linker of (a) takes place concomitantly. In another embodiment, the chemical moiety of (b) that can be used to isolate the attached oligonucleotide sequence comprises a trityl group, for example a dimethoxytrityl group, which can be employed in a trityl-on synthesis strategy as described herein. In yet another embodiment, the chemical moiety, such as a dimethoxytrityl group, is removed during purification, for example, using acidic conditions.

In a further embodiment, the method for siNA synthesis is a solution phase synthesis or hybrid phase synthesis wherein both strands of the siNA duplex are synthesized in tandem using a cleavable linker attached to the first sequence which acts a scaffold for synthesis of the second sequence. Cleavage of the linker under conditions suitable for hybridization of the separate siNA sequence strands results in formation of the double-stranded siNA molecule.

In another embodiment, the invention features a method for synthesizing a siNA duplex molecule comprising: (a) synthesizing one oligonucleotide sequence strand of the

siNA molecule, wherein the sequence comprises a cleavable linker molecule that can be used as a scaffold for the synthesis of another oligonucleotide sequence; (b) synthesizing a second oligonucleotide sequence having complementarity to the first sequence strand on the scaffold of (a), wherein the second sequence comprises the other strand of the double-stranded siNA molecule and wherein the second sequence further comprises a chemical moiety than can be used to isolate the attached oligonucleotide sequence; (c) purifying the product of (b) utilizing the chemical moiety of the second oligonucleotide sequence strand under conditions suitable for isolating the full-length sequence comprising both siNA oligonucleotide strands connected by the cleavable linker and under conditions suitable for the two siNA oligonucleotide strands to hybridize and form a stable duplex. In one embodiment, cleavage of the linker molecule in (c) above takes place during deprotection of the oligonucleotide, for example under hydrolysis conditions. In another embodiment, cleavage of the linker molecule in (c) above takes place after deprotection of the oligonucleotide. In another embodiment, the method of synthesis comprises solid phase synthesis on a solid support such as controlled pore glass (CPG) or polystyrene, wherein the first sequence of (a) is synthesized on a cleavable linker, such as a succinyl linker, using the solid support as a scaffold. The cleavable linker in (a) used as a scaffold for synthesizing the second strand can comprise similar reactivity or differing reactivity as the solid support derivatized linker, such that cleavage of the solid support derivatized linker and the cleavable linker of (a) takes place either concomitantly or sequentially. In one embodiment, the chemical moiety of (b) that can be used to isolate the attached oligonucleotide sequence comprises a trityl group, for example a dimethoxytrityl group.

In another embodiment, the invention features a method for making a double-stranded siNA molecule in a single synthetic process comprising: (a) synthesizing an oligonucleotide having a first and a second sequence, wherein the first sequence is complementary to the second sequence, and the first oligonucleotide sequence is linked to the second sequence via a cleavable linker, and wherein a terminal 5'-protecting group, for example, a 5'-O-dimethoxytrityl group (5'-O-DMT) remains on the oligonucleotide having the second sequence; (b) deprotecting the oligonucleotide whereby the deprotection results in the cleavage of the linker joining the two oligonucleotide sequences; and (c) purifying the product of (b) under conditions suitable for isolating the

double-stranded siNA molecule, for example using a trityl-on synthesis strategy as described herein.

In another embodiment, the method of synthesis of siNA molecules of the invention comprises the teachings of Scaringe *et al.*, US Patent Nos. 5,889,136; 6,008,400; and 6,111,086, incorporated by reference herein in their entirety.

In one embodiment, the invention features siNA constructs that mediate RNAi in a cell or reconstituted system, wherein the siNA construct comprises one or more chemical modifications, for example, one or more chemical modifications having any of Formulae I-VII or any combination thereof that increases the nuclease resistance of the siNA construct.

In another embodiment, the invention features a method for generating siNA molecules with increased nuclease resistance comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased nuclease resistance.

In one embodiment, the invention features siNA constructs that mediate RNAi against a target gene, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the sense and antisense strands of the siNA construct.

In another embodiment, the invention features a method for generating siNA molecules with increased binding affinity between the sense and antisense strands of the siNA molecule comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased binding affinity between the sense and antisense strands of the siNA molecule.

In one embodiment, the invention features siNA constructs that mediate RNAi in a cell or reconstituted system, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the antisense strand of the siNA construct and a complementary target RNA sequence within a cell.

In one embodiment, the invention features siNA constructs that mediate RNAi in a cell or reconstituted system, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the binding affinity between the antisense strand of the siNA construct and a complementary target DNA sequence within a cell.

In another embodiment, the invention features a method for generating siNA molecules with increased binding affinity between the antisense strand of the siNA molecule and a complementary target RNA sequence comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased binding affinity between the antisense strand of the siNA molecule and a complementary target RNA sequence.

In another embodiment, the invention features a method for generating siNA molecules with increased binding affinity between the antisense strand of the siNA molecule and a complementary target DNA sequence comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having increased binding affinity between the antisense strand of the siNA molecule and a complementary target DNA sequence.

In one embodiment, the invention features siNA constructs that mediate RNAi in a cell or reconstituted system, wherein the siNA construct comprises one or more chemical modifications described herein that modulate the polymerase activity of a cellular polymerase capable of generating additional endogenous siNA molecules having sequence homology to the chemically-modified siNA construct.

In another embodiment, the invention features a method for generating siNA molecules capable of mediating increased polymerase activity of a cellular polymerase capable of generating additional endogenous siNA molecules having sequence homology to a chemically-modified siNA molecule comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules capable of mediating increased polymerase activity of a cellular polymerase capable of generating additional endogenous siNA molecules having sequence homology to the

chemically-modified siNA molecule. In one embodiment, the invention features chemically-modified siNA constructs that mediate RNAi in a cell or reconstituted system, wherein the chemical modifications do not significantly effect the interaction of siNA with a target RNA molecule, DNA molecule and/or proteins or other factors that are essential for RNAi in a manner that would decrease the efficacy of RNAi mediated by such siNA constructs.

In another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi activity.

In yet another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against a target RNA comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi activity against the target RNA.

In yet another embodiment, the invention features a method for generating siNA molecules with improved RNAi activity against a DNA target comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved RNAi activity against the DNA target, such as a gene, chromosome, or portion thereof.

In one embodiment, the invention features siNA constructs that mediate RNAi in a cell or reconstituted system, wherein the siNA construct comprises one or more chemical modifications described herein that modulates the cellular uptake of the siNA construct.

In another embodiment, the invention features a method for generating siNA molecules against a target gene with improved cellular uptake comprising (a) introducing nucleotides having any of Formula I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved cellular uptake.

In one embodiment, the invention features siNA constructs that mediate RNAi against a target gene, wherein the siNA construct comprises one or more chemical modifications described herein that increases the bioavailability of the siNA construct, for example, by attaching polymeric conjugates such as polyethyleneglycol or equivalent conjugates that improve the pharmacokinetics of the siNA construct, or by attaching conjugates that target specific tissue types or cell types *in vivo*. Non-limiting examples of such conjugates are described in Vargeese *et al.*, U.S. Serial No. 10/201,394 incorporated by reference herein.

In one embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability, comprising (a) introducing a conjugate into the structure of a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability. Such conjugates can include ligands for cellular receptors, such as peptides derived from naturally occurring protein ligands; protein localization sequences, including cellular ZIP code sequences; antibodies; nucleic acid aptamers; vitamins and other co-factors, such as folate and N-acetylgalactosamine; polymers, such as polyethyleneglycol (PEG); phospholipids; polyamines, such as spermine or spermidine; and others.

In another embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability comprising (a) introducing an excipient formulation to a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability. Such excipients include polymers such as cyclodextrins, lipids, cationic lipids, polyamines, phospholipids, and others.

In another embodiment, the invention features a method for generating siNA molecules of the invention with improved bioavailability comprising (a) introducing nucleotides having any of Formulae I-VII or any combination thereof into a siNA molecule, and (b) assaying the siNA molecule of step (a) under conditions suitable for isolating siNA molecules having improved bioavailability.

In another embodiment, polyethylene glycol (PEG) can be covalently attached to siNA compounds of the present invention. The attached PEG can be any molecular weight, preferably from about 2,000 to about 50,000 daltons (Da).

The present invention can be used alone or as a component of a kit having at least one of the reagents necessary to carry out the *in vitro* or *in vivo* introduction of RNA to test samples and/or subjects. For example, preferred components of the kit include a siNA molecule of the invention and a vehicle that promotes introduction of the siNA into cells of interest as described herein (e.g., using lipids and other methods of transfection known in the art, see for example Beigelman *et al,* US 6,395,713). The kit can be used for target validation, such as in determining gene function and/or activity, or in drug optimization, and in drug discovery (see for example Usman et al., USSN 60/402,996). Such a kit can also include instructions to allow a user of the kit to practice the invention.

The term "short interfering nucleic acid", "siNA", "short interfering RNA", "siRNA", "short interfering nucleic acid molecule", "short interfering oligonucleotide molecule", or "chemically-modified short interfering nucleic acid molecule" as used herein refers to any nucleic acid molecule capable of inhibiting or down regulating gene expression or viral replication, for example by mediating RNA interference "RNAi" or gene silencing in a sequence-specific manner; see for example Bass, 2001, *Nature*, 411, 428-429; Elbashir *et al.,* 2001, *Nature*, 411, 494-498; and Kreutzer *et al.,* International PCT Publication No. WO 00/44895; Zernicka-Goetz *et al.,* International PCT Publication No. WO 01/36646; Fire, International PCT Publication No. WO 99/32619; Plaetinck *et al.,* International PCT Publication No. WO 00/01846; Mello and Fire, International PCT Publication No. WO 01/29058; Deschamps-Depaillette, International PCT Publication No. WO 99/07409; and Li *et al.,* International PCT Publication No. WO 00/44914; Allshire, 2002, *Science,* 297, 1818-1819; Volpe *et al.,* 2002, *Science,* 297, 1833-1837; Jenuwein, 2002, *Science,* 297, 2215-2218; and Hall *et al.,* 2002, *Science,* 297, 2232-2237; Hutvagner and Zamore, 2002, *Science*, 297, 2056-60; McManus *et al.,* 2002, *RNA*, 8, 842-850; Reinhart *et al.,* 2002, *Gene & Dev.,* 16, 1616-1626; and Reinhart & Bartel, 2002, *Science*, 297, 1831). Non limiting examples of siNA molecules of the invention are shown in **Figures 18-20,** and **Tables II, III, and IV** herein. For example the siNA can be a double-stranded polynucleotide molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be assembled from two separate

oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary (i.e. each strand comprises nucleotide sequence that is complementary to nucleotide sequence in the other strand; such as where the antisense strand and sense strand form a duplex or double stranded structure, for example wherein the double stranded region is about 19 base pairs); the antisense strand comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense strand comprises nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. Alternatively, the siNA is assembled from a single oligonucleotide, where the self-complementary sense and antisense regions of the siNA are linked by means of a nucleic acid based or non-nucleic acid-based linker(s). The siNA can be a polynucleotide with a hairpin secondary structure, having self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a separate target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The siNA can be a circular single-stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof, and wherein the circular polynucleotide can be processed either *in vivo* or *in vitro* to generate an active siNA molecule capable of mediating RNAi. The siNA can also comprise a single stranded polynucleotide having nucleotide sequence complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof (for example, where such siNA molecule does not require the presence within the siNA molecule of nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof), wherein the single stranded polynucleotide can further comprise a terminal phosphate group, such as a 5'-phosphate (see for example Martinez *et al.*, 2002, *Cell.*, 110, 563-574 and Schwarz *et al.*, 2002, *Molecular Cell*, 10, 537-568), or 5',3'-diphosphate. In certain embodiment, the siNA molecule of the invention comprises separate sense and antisense sequences or regions, wherein the sense and antisense regions are covalently linked by nucleotide or non-nucleotide linkers molecules as is known in the art, or are alternately

non-covalently linked by ionic interactions, hydrogen bonding, van der waals interactions, hydrophobic intercations, and/or stacking interactions. In certain embodiments, the siNA molecules of the invention comprise nucleotide sequence that is complementary to nucleotide sequence of a target gene. In another embodiment, the siNA molecule of the invention interacts with nucleotide sequence of a target gene in a manner that causes inhibition of expression of the target gene. As used herein, siNA molecules need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides. In certain embodiments, the short interfering nucleic acid molecules of the invention lack 2'-hydroxy (2'-OH) containing nucleotides. Applicant describes in certain embodiments short interfering nucleic acids that do not require the presence of nucleotides having a 2'-hydroxy group for mediating RNAi and as such, short interfering nucleic acid molecules of the invention optionally do not include any ribonucleotides (e.g., nucleotides having a 2'-OH group). Such siNA molecules that do not require the presence of ribonucleotides within the siNA molecule to support RNAi can however have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. Optionally, siNA molecules can comprise ribonucleotides at about 5, 10, 20, 30, 40, or 50% of the nucleotide positions. The modified short interfering nucleic acid molecules of the invention can also be referred to as short interfering modified oligonucleotides "siMON." As used herein, the term siNA is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi, for example short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid, short interfering modified oligonucleotide, chemically-modified siRNA, post-transcriptional gene silencing RNA (ptgsRNA), and others. In addition, as used herein, the term RNAi is meant to be equivalent to other terms used to describe sequence specific RNA interference, such as post transcriptional gene silencing, or epigenetics. For example, siNA molecules of the invention can be used to epigenetically silence genes at both the post-transcriptional level or the pre-transcriptional level. In a non-limiting example, epigenetic regulation of gene expression by siNA molecules of the invention can result from siNA mediated modification of chromatin structure to alter gene expression (see, for example, Allshire,

2002, *Science,* 297, 1818-1819; Volpe *et al.,* 2002, *Science,* 297, 1833-1837; Jenuwein, 2002, *Science,* 297, 2215-2218; and Hall *et al.,* 2002, *Science,* 297, 2232-2237).

By "modulate" is meant that the expression of the gene, or level of RNA molecule or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits is up regulated or down regulated, such that expression, level, or activity is greater than or less than that observed in the absence of the modulator. For example, the term "modulate" can mean "inhibit," but the use of the word "modulate" is not limited to this definition.

By "inhibit", "down-regulate", or "reduce", it is meant that the expression of the gene, or level of RNA molecules or equivalent RNA molecules encoding one or more proteins or protein subunits, or activity of one or more proteins or protein subunits, is reduced below that observed in the absence of the nucleic acid molecules (e.g., siNA) of the invention. In one embodiment, inhibition, down-regulation or reduction with an siNA molecule is below that level observed in the presence of an inactive or attenuated molecule. In another embodiment, inhibition, down-regulation, or reduction with siNA molecules is below that level observed in the presence of, for example, an siNA molecule with scrambled sequence or with mismatches. In another embodiment, inhibition, down-regulation, or reduction of gene expression with a nucleic acid molecule of the instant invention is greater in the presence of the nucleic acid molecule than in its absence.

By "gene" or "target gene" is meant, a nucleic acid that encodes an RNA, for example, nucleic acid sequences including, but not limited to, structural genes encoding a polypeptide. The target gene can be a gene derived from a cell, an endogenous gene, a transgene, or exogenous genes such as genes of a pathogen, for example a virus, which is present in the cell after infection thereof. The cell containing the target gene can be derived from or contained in any organism, for example a plant, animal, protozoan, virus, bacterium, or fungus. Non-limiting examples of plants include monocots, dicots, or gymnosperms. Non-limiting examples of animals include vertebrates or invertebrates. Non-limiting examples of fungi include molds or yeasts.

By "highly conserved sequence region" is meant, a nucleotide sequence of one or more regions in a target gene does not vary significantly from one generation to the other or from one biological system to the other.

By "cancer" is meant a group of diseases characterized by uncontrolled growth and spread of abnormal cells.

By "sense region" is meant a nucleotide sequence of a siNA molecule having complementarity to an antisense region of the siNA molecule. In addition, the sense region of a siNA molecule can comprise a nucleic acid sequence having homology with a target nucleic acid sequence.

By "antisense region" is meant a nucleotide sequence of a siNA molecule having complementarity to a target nucleic acid sequence. In addition, the antisense region of a siNA molecule can optionally comprise a nucleic acid sequence having complementarity to a sense region of the siNA molecule.

By "target nucleic acid" is meant any nucleic acid sequence whose expression or activity is to be modulated. The target nucleic acid can be DNA or RNA.

By "complementarity" is meant that a nucleic acid can form hydrogen bond(s) with another nucleic acid sequence by either traditional Watson-Crick or other non-traditional types. In reference to the nucleic molecules of the present invention, the binding free energy for a nucleic acid molecule with its complementary sequence is sufficient to allow the relevant function of the nucleic acid to proceed, e.g., RNAi activity. Determination of binding free energies for nucleic acid molecules is well known in the art (see, e.g., Turner *et al.*, 1987, *CSH Symp. Quant. Biol.* LII pp.123-133; Frier *et al.*, 1986, *Proc. Nat. Acad. Sci.* USA 83:9373-9377; Turner *et al.*, 1987, *J. Am. Chem. Soc.* 109:3783-3785). A percent complementarity indicates the percentage of contiguous residues in a nucleic acid molecule that can form hydrogen bonds (e.g., Watson-Crick base pairing) with a second nucleic acid sequence (e.g., 5, 6, 7, 8, 9, 10 out of 10 being 50%, 60%, 70%, 80%, 90%, and 100% complementary). "Perfectly complementary" means that all the contiguous residues of a nucleic acid sequence will hydrogen bond with the same number of contiguous residues in a second nucleic acid sequence.

The siNA molecules of the invention represent a novel therapeutic approach to a broad spectrum of diseases and conditions, including cancer or cancerous disease, infectious disease, cardiovascular disease, neurological disease, prion disease, inflammatory disease, autoimmune disease, pulmonary disease, renal disease, liver

disease, mitochondrial disease, endocrine disease, reproduction related diseases and conditions, and any other indications that can respond to the level of an expressed gene product in a cell or organsim.

In one embodiment of the present invention, each sequence of a siNA molecule of the invention is independently about 18 to about 24 nucleotides in length, in specific embodiments about 18, 19, 20, 21, 22, 23, or 24 nucleotides in length. In another embodiment, the siNA duplexes of the invention independently comprise about 17 to about 23 base pairs (*e.g.*, about 17, 18, 19, 20, 21, 22 or 23). In yet another embodiment, siNA molecules of the invention comprising hairpin or circular structures are about 35 to about 55 (*e.g.*, about 35, 40, 45, 50 or 55) nucleotides in length, or about 38 to about 44 (*e.g.*, 38, 39, 40, 41, 42, 43 or 44) nucleotides in length and comprising about 16 to about 22 (*e.g.*, about 16, 17, 18, 19, 20, 21 or 22) base pairs. Exemplary siNA molecules of the invention are shown in **Table II**. Exemplary synthetic siNA molecules of the invention are shown in **Table I** and/or **Figures 18-19**.

As used herein "cell" is used in its usual biological sense, and does not refer to an entire multicellular organism, e.g., specifically does not refer to a human. The cell can be present in an organism, e.g., birds, plants and mammals such as humans, cows, sheep, apes, monkeys, swine, dogs, and cats. The cell can be prokaryotic or eukaryotic (e.g., mammalian or plant cell). The cell can be of somatic or germ line origin, totipotent or pluripotent, dividing or non-dividing. The cell can also be derived from or can comprise a gamete or embryo, a stem cell, or a fully differentiated cell.

The siNA molecules of the invention are added directly, or can be complexed with cationic lipids, packaged within liposomes, or otherwise delivered to target cells or tissues. The nucleic acid or nucleic acid complexes can be locally administered to relevant tissues *ex vivo*, or *in vivo* through injection, infusion pump or stent, with or without their incorporation in biopolymers. In particular embodiments, the nucleic acid molecules of the invention comprise sequences shown in **Tables I-II** and/or **Figures 18-19**. Examples of such nucleic acid molecules consist essentially of sequences defined in these tables and figures. Furthermore, the chemically modified constructs described in **Table IV** can be applied to any siNA sequence of the invention.

In another aspect, the invention provides mammalian cells containing one or more siNA molecules of this invention. The one or more siNA molecules can independently be targeted to the same or different sites.

By "RNA" is meant a molecule comprising at least one ribonucleotide residue. By "ribonucleotide" is meant a nucleotide with a hydroxyl group at the 2' position of a β-D-ribo-furanose moiety. The terms include double-stranded RNA, single-stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the siNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in the RNA molecules of the instant invention can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA.

By "subject" is meant an organism, which is a donor or recipient of explanted cells or the cells themselves. "Subject" also refers to an organism to which the nucleic acid molecules of the invention can be administered. In one embodiment, a subject is a mammal or mammalian cells. In another embodiment, a subject is a human or human cells.

The term "phosphorothioate" as used herein refers to an internucleotide linkage having Formula I, wherein Z and/or W comprise a sulfur atom. Hence, the term phosphorothioate refers to both phosphorothioate and phosphorodithioate internucleotide linkages.

The term "universal base" as used herein refers to nucleotide base analogs that form base pairs with each of the natural DNA/RNA bases with little discrimination between them. Non-limiting examples of universal bases include C-phenyl, C-naphthyl and other aromatic derivatives, inosine, azole carboxamides, and nitroazole derivatives such as 3-nitropyrrole, 4-nitroindole, 5-nitroindole, and 6-nitroindole as known in the art (see for example Loakes, 2001, *Nucleic Acids Research,* 29, 2437-2447).

The term "acyclic nucleotide" as used herein refers to any nucleotide having an acyclic ribose sugar, for example where any of the ribose carbons (C1, C2, C3, C4, or C5), are independently or in combination absent from the nucleotide.

The nucleic acid molecules of the instant invention, individually, or in combination or in conjunction with other drugs, can be used to treat diseases or conditions discussed herein. For example, to treat a particular disease or condition, the siNA molecules can be administered to a subject or can be administered to other appropriate cells evident to those skilled in the art, individually or in combination with one or more drugs under conditions suitable for the treatment.

In a further embodiment, the siNA molecules can be used in combination with other known treatments to treat conditions or diseases discussed above. For example, the described molecules could be used in combination with one or more known therapeutic agents to treat a disease or condition. Non-limiting examples of other therapeutic agents that can be readily combined with a siNA molecule of the invention are enzymatic nucleic acid molecules, allosteric nucleic acid molecules, antisense, decoy, or aptamer nucleic acid molecules, antibodies such as monoclonal antibodies, small molecules, and other organic and/or inorganic compounds including metals, salts and ions.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a non-limiting example of a scheme for the synthesis of siNA molecules. The complementary siNA sequence strands, strand 1 and strand 2, are synthesized in tandem and are connected by a cleavable linkage, such as a nucleotide succinate or abasic succinate, which can be the same or different from the cleavable linker used for solid phase synthesis on a solid support. The synthesis can be either solid phase or solution phase, in the example shown, the synthesis is a solid phase synthesis. The synthesis is performed such that a protecting group, such as a dimethoxytrityl group, remains intact on the terminal nucleotide of the tandem oligonucleotide. Upon cleavage and deprotection of the oligonucleotide, the two siNA strands spontaneously hybridize to form a siNA duplex, which allows the purification of the duplex by utilizing the

properties of the terminal protecting group, for example by applying a trityl on purification method wherein only duplexes/oligonucleotides with the terminal protecting group are isolated.

Figure 2 shows a MALDI-TOV mass spectrum of a purified siNA duplex synthesized by a method of the invention. The two peaks shown correspond to the predicted mass of the separate siNA sequence strands. This result demonstrates that the siNA duplex generated from tandem synthesis can be purified as a single entity using a simple trityl-on purification methodology.

Figure 3 shows the results of a stability assay used to determine the serum stability of chemically modified siNA constructs compared to a siNA control consisting of all RNA with 3'-TT termini. T ½ values are shown for duplex stability.

Figure 4 shows the results of an RNAi activity screen of several phosphorothioate modified siNA constructs using a luciferase reporter system.

Figure 5 shows the results of an RNAi activity screen of several phosphorothioate and universal base modified siNA constructs using a luciferase reporter system.

Figure 6 shows the results of an RNAi activity screen of several 2'-O-methyl modified siNA constructs using a luciferase reporter system.

Figure 7 shows the results of an RNAi activity screen of several 2'-O-methyl and 2'-deoxy-2'-fluoro modified siNA constructs using a luciferase reporter system.

Figure 8 shows the results of an RNAi activity screen of a phosphorothioate modified siNA construct using a luciferase reporter system.

Figure 9 shows the results of an RNAi activity screen of an inverted deoxyabasic modified siNA construct generated via tandem synthesis using a luciferase reporter system.

Figure 10 shows the results of an RNAi activity screen of chemically modifed siNA constructs including 3'-glyceryl modified siNA constructs compared to an all RNA control siNA construct using a luciferase reporter system. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM

concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by RPI number (sense strand/antisense strand). Sequences correspoding to these RPI numbers are shown in Table I.

Figure 11 shows the results of an RNAi activity screen of chemically modifed siNA constructs. The screen compared various combinations of sense strand chemical modifications and antisense strand chemical modifications. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by RPI number (sense strand/antisense strand). Sequences correspoding to these RPI numbers are shown in Table I.

Figure 12 shows the results of an RNAi activity screen of chemically modifed siNA constructs. The screen compared various combinations of sense strand chemical modifications and antisense strand chemical modifications. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by RPI number (sense strand/antisense strand). Sequences correspoding to these RPI numbers are shown in Table I. In addition, the antisense strand alone (RPI 30430) and an inverted control (RPI 30227/30229, having matched chemistry to RPI (30063/30224) was compared to the siNA duplexes described above.

Figure 13 shows the results of an RNAi activity screen of chemically modifed siNA constructs. The screen compared various combinations of sense strand chemical modifications and antisense strand chemical modifications. These chemically modified

siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by RPI number (sense strand/antisense strand). Sequences correspoding to these RPI numbers are shown in Table I. In addition, an inverted control (RPI 30226/30229), having matched chemistry to RPI (30222/30224) was compared to the siNA duplexes described above.

Figure 14 shows the results of an RNAi activity screen of chemically modifed siNA constructs including various 3'-terminal modified siNA constructs compared to an all RNA control siNA construct using a luciferase reporter system. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by RPI number (sense strand/antisense strand). Sequences correspoding to these RPI numbers are shown in Table I.

Figure 15 shows the results of an RNAi activity screen of chemically modifed siNA constructs. The screen compared various combinations of sense strand chemistries compared to a fixed antisense strand chemistry. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by RPI number (sense strand/antisense strand). Sequences correspoding to these RPI numbers are shown in Table I.

Figure 16 shows the results of a siNA titration study using a luciferase reporter system, wherein the RNAi activity of a phosphorothioate modified siNA construct is

compared to that of a siNA construct consisting of all ribonucleotides except for two terminal thymidine residues.

Figure 17 shows a non-limiting proposed mechanistic representation of target RNA degradation involved in RNAi. Double-stranded RNA (dsRNA), which is generated by RNA-dependent RNA polymerase (RdRP) from foreign single-stranded RNA, for example viral, transposon, or other exogenous RNA, activates the DICER enzyme that in turn generates siNA duplexes. Alternately, synthetic or expressed siNA can be introduced directely into a cell by appropriate means. An active siNA complex forms which recognizes a target RNA, resulting in degradation of the target RNA by the RISC endonuclease complex or in the synthesis of additional RNA by RNA-dependent RNA polymerase (RdRP), which can activate DICER and result in additional siNA molecules, thereby amplifying the RNAi response.

Figure 18A-F shows non-limiting examples of chemically-modified siNA constructs of the present invention. In the figure, N stands for any nucleotide (adenosine, guanosine, cytosine, uridine, or optionally thymidine, for example thymidine can be substituted in the overhanging regions designated by parenthesis (N N). Various modifications are shown for the sense and antisense strands of the siNA constructs.

Figure 18A: The sense strand comprises 21 nucleotides having four phosphorothioate 5'- and 3'-terminal internucleotide linkages, wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-O-methyl or 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and having one 3'-terminal phosphorothioate internucleotide linkage and four 5'-terminal phosphorothioate internucleotide linkages and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein.

Figure 18B: The sense strand comprises 21 nucleotides wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-O-methyl or 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein.

Figure 18C: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-O-methyl or 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and having one 3'-terminal phosphorothioate internucleotide linkage and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein.

Figure 18D: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein and wherein and all purine nucleotides that may be present are 2'-deoxy nucleotides. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and having one 3'-terminal phosphorothioate internucleotide linkage and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified

nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein.

Figure 18E: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-O-methyl modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein.

Figure 18F: The sense strand comprises 21 nucleotides having 5'- and 3'- terminal cap moieties wherein the two terminal 3'-nucleotides are optionally base paired and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand comprises 21 nucleotides, optionally having a 3'-terminal glyceryl moiety and wherein the two terminal 3'-nucleotides are optionally complementary to the target RNA sequence, and having one 3'-terminal phosphorothioate internucleotide linkage and wherein all pyrimidine nucleotides that may be present are 2'-deoxy-2'-fluoro modified nucleotides and all purine nucleotides that may be present are 2'-deoxy modified nucleotides except for (N N) nucleotides, which can comprise ribonucleotides, deoxynucleotides, universal bases, or other chemical modifications described herein. The antisense strand of constructs A-F comprise sequence complementary to target RNA sequence of the invention.

Figure 19 shows non-limiting examples of specific chemically modified siNA sequences of the invention. **A-F** applies the chemical modifications described in **Figure 18A-F** to a representative siNA sequence targeting the hepatitis C virus (HCV).

Figure 20 shows non-limiting examples of different siNA constructs of the invention. The examples shown (constructs 1, 2, and 3) have 19 representative base pairs, however, different embodiments of the invention include any number of base pairs described herein. Bracketed regions represent nucleotide overhangs, for example comprising about 1, 2, 3, or 4 nucleotides in length, preferably about 2 nucleotides. Constructs 1 and 2 can be used independently for RNAi activity. Construct 2 can comprise a polynucleotide or non-nucleotide linker, which can optionally be designed as a biodegradable linker. In one embodiment, the loop structure shown in construct 2 can comprise a biodegradable linker that results in the formation of construct 1 in vivo and/or in vitro. In another example, construct 3 can be used to generate construct 2 under the same principle wherein a linker is used to generate the active siNA construct 2 in vivo and/or in vitro, which can optionally utilize another biodegradable linker to generate the active siNA construct 1 in vivo and/or in vitro. As such, the stability and/or activity of the siNA constructs can be modulated based on the design of the siNA construct for use in vivo or in vitro and/or in vitro.

Figure 21 is a diagrammatic representation of a method used to determine target sites for siNA mediated RNAi within a particular target nucleic acid sequence, such as messenger RNA. (A) A pool of siNA oligonucleotides are synthesized wherein the antisense region of the siNA constructs has complementarity to target sites across the target nucleic acid sequence, and wherein the sense region comprises sequence complementary to the antisense region of the siNA. (B) The sequences are transfected into cells. (C) Cells are selected based on phenotypic change that is associated with modulation of the target nucleic acid sequence. (D) The siNA is isolated from the selected cells and is sequenced to identify efficacious target sites within the target nucleic acid sequence.

Figure 22 shows non-limiting examples of different stabilization chemistries (1-10) that can be used, for example, to stabilize the 3'-end of siNA sequences of the invention, including **(1)** [3-3']-inverted deoxyribose; **(2)** deoxyribonucleotide; **(3)** [5'-3']-3'-

deoxyribonucleotide; **(4)** [5'-3']-ribonucleotide; **(5)** [5'-3']-3'-O-methyl ribonucleotide; **(6)** 3'-glyceryl; **(7)** [3'-5']-3'-deoxyribonucleotide; **(8)** [3'-3']-deoxyribonucleotide; **(9)** [5'-2']-deoxyribonucleotide; and **(10)** [5-3']-dideoxyribonucleotide. In addition to modified and unmodified backbone chemistries indicated in the figure, these chemistries can be combined with different backbone modifications as described herein, for example, backbone modifications having Formula I. In addition, the 2'-deoxy nucleotide shown 5' to the terminal modifications shown can be another modified or unmodified nucleotide or non-nucleotide described herein, for example modifications having any of Formulae I-VII or any combination thereof.

Figure 23 shows a non-limiting example of siNA mediated inhibition of VEGF-induced angiogenesis using the rat corneal model of angiogenesis. siNA targeting site 2340 of VEGFR1 RNA (shown as RPI No. 29695/29699) were compared to inverted controls (shown as RPI No. 29983/29984) at three different concentrations and compared to a VEGF control in which no siNA was administered.

Figure 24 is a non-limiting example of a HBsAg screen of stabilized siNA constructs ("stab 4/5", see **Table IV**) targeting HBV pregenomic RNA in HepG2 cells at 25 nM compared to untreated and matched chemistry inverted sequence controls. The siNA sense and antisense strands are shown by RPI number (sense/antisense).

Figure 25 is a non-limiting example of a dose response HBsAg screen of stabilized siNA constructs ("stab 4/5", see **Table IV**) targeting sites 262 and 1580 of the HBV pregenomic RNA in HepG2 cells at 0.5, 5, 10 and 25 nM compared to untreated and matched chemistry inverted sequence controls. The siNA sense and antisense strands are shown by RPI number (sense/antisense).

Figure 26 shows a dose response comparison of two different stabilization chemistries ("stab 7/8" and "stab 7/11", see **Table IV**) targeting site 1580 of the HBV pregenomic RNA in HepG2 cells at 5, 10, 25, 50 and 100 nM compared to untreated and matched chemistry inverted sequence controls. The siNA sense and antisense strands are shown by RPI number (sense/antisense).

Figure 27 shows a non-limiting example of a strategy used to identify chemically modified siNA constructs of the invention that are nuclease resistance while preserving

the ability to mediate RNAi activity. Chemical modifications are introduced into the siNA construct based on educated design parameters (e.g. introducing 2'-mofications, base modifications, backbone modifications, terminal cap modifications etc). The modified construct in tested in an appropriate system (e.g human serum for nuclease resistance, shown, or an animal model for PK/delivery parameters). In parallel, the siNA construct is tested for RNAi activity, for example in a cell culture system such as a luciferase reporter assay). Lead siNA constructs are then identified which possess a particular characteristic while maintaining RNAi activity, and can be further modified and assayed once again. This same approach can be used to identify siNA-conjugate molecules with improved pharmacokinetic profiles, delivery, and RNAi activity.

Figure 28 shows representative data of a chemically modified siNA construct (Stab 4/5, **Table IV**) targeting HBV site 1580 RNA compared to an unstabilized siRNA construct in a dose response time course HBsAg assay. The constructs were compared at different concentrations (5nM, 10 nM, 25 nM, 50 nM, and 100 nM) over the course of nine days. Activity based on HBsAg levels was determined at day 3, day 6, and day 9.

Figure 29 shows representative data of a chemically modified siNA construct (Stab 7/8, **Table IV**) targeting HBV site 1580 RNA compared to an unstabilized siRNA construct in a dose response time course HBsAg assay. The constructs were compared at different concentrations (5nM, 10 nM, 25 nM, 50 nM, and 100 nM) over the course of nine days. SiNA activity based on HBsAg levels was determined at day 3, day 6, and day 9.

Figure 30 shows representative data of a chemically modified siNA construct (Stab 7/11, **Table IV**) targeting HBV site 1580 RNA compared to an unstabilized siRNA construct in a dose response time course HBsAg assay. The constructs were compared at different concentrations (5nM, 10 nM, 25 nM, 50 nM, and 100 nM) over the course of nine days. SiNA activity based on HBsAg levels was determined at day 3, day 6, and day 9.

Figure 31 shows representative data of a chemically modified siNA construct (Stab 9/10, **Table IV**) targeting HBV site 1580 RNA compared to an unstabilized siRNA construct in a dose response time course HBsAg assay. The constructs were compared at different concentrations (5nM, 10 nM, 25 nM, 50 nM, and 100 nM) over the course of

nine days. SiNA activity based on HBsAg levels was determined at day 3, day 6, and day 9.

Figure 32 shows non-limiting examples of inhibition of viral replication of a HCV/poliovirus chimera by siNA constructs targeted to HCV chimera (29579/29586; 29578/29585) compared to control (29593/29600).

Figure 33 shows a non-limiting example of a dose response study demonstrating the inhibition of viral replication of a HCV/poliovirus chimera by siNA construct (29579/29586) at various concentrations (1nM, 5nM, 10nM, and 25nM) compared to control (29593/29600).

Figure 34 shows a non-limiting example demonstrating the inhibition of viral replication of a HCV/poliovirus chimera by a chemically modified siRNA construct (30051/30053) compared to control construct (30052/30054).

Figure 35 shows a non-limiting example demonstrating the inhibition of viral replication of a HCV/poliovirus chimera by a chemically modified siRNA construct (30055/30057) compared to control construct (30056/30058).**Figure 36** shows a non-limiting example of several chemically modified siRNA constructs targeting viral replication of an HCV/poliovirus chimera at 10 nM treatment in comparison to a lipid control and an inverse siNA control construct 29593/ 29600.

Figure 37 shows a non-limiting example of several chemically modified siRNA constructs targeting viral replication of a HCV/poliovirus chimera at 25 nM treatment in comparison to a lipid control and an inverse siNA control construct 29593/ 29600.

Figure 38 shows a non-limiting example of several chemically modified siRNA constructs targeting viral replication of a Huh7 HCV replicon system at 25 nM treatment in comparison to untreated cells ("cells"), cells transfected with lipofectamine ("LFA2K") and inverse siNA control constructs.

DETAILED DESCRIPTION OF THE INVENTION

Mechanism of action of Nucleic Acid Molecules of the Invention

The discussion that follows discusses the proposed mechanism of RNA interference mediated by short interfering RNA as is presently known, and is not meant to be limiting and is not an admission of prior art. Applicant demonstrates herein that chemically-modified short interfering nucleic acids possess similar or improved capacity to mediate RNAi as do siRNA molecules and are expected to possess improved stability and activity *in vivo*; therefore, this discussion is not meant to be limiting only to siRNA and can be applied to siNA as a whole. By "improved capacity to mediate RNAi" or "improved RNAi activity" is meant to include RNAi activity measured *in vitro* and/or *in vivo* where the RNAi activity is a reflection of both the ability of the siNA to mediate RNAi and the stability of the siNAs of the invention. In this invention, the product of these activities can be increased *in vitro* and/or *in vivo* compared to an all RNA siRNA or a siNA containing a plurality of ribonucleotides. In some cases, the activity or stability of the siNA molecule can be decreased (i.e., less than ten-fold), but the overall activity of the siNA molecule is enhanced *in vitro* and/or *in vivo*.

RNA interference refers to the process of sequence specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs) (Fire *et al.*, 1998, *Nature,* 391, 806). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes which is commonly shared by diverse flora and phyla (Fire *et al.*, 1999, *Trends Genet.*, 15, 358). Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA. The presence of dsRNA in cells triggers the RNAi response though a mechanism that has yet to be fully characterized. This mechanism appears to be different from the interferon response that results from dsRNA-mediated activation of protein kinase PKR and 2', 5'-oligoadenylate synthetase resulting in non-specific cleavage of mRNA by ribonuclease L.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as Dicer. Dicer is involved in the processing of the dsRNA into short

pieces of dsRNA known as short interfering RNAs (siRNAs) (Berstein *et al.*, 2001, *Nature,* 409, 363). Short interfering RNAs derived from Dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. Dicer has also been implicated in the excision of 21- and 22-nucleotide small temporal RNAs (stRNAs) from precursor RNA of conserved structure that are implicated in translational control (Hutvagner *et al.*, 2001, *Science,* 293, 834). The RNAi response also features an endonuclease complex containing a siRNA, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence homologous to the siRNA. Cleavage of the target RNA takes place in the middle of the region complementary to the guide sequence of the siRNA duplex (Elbashir *et al.*, 2001, *Genes Dev.*, 15, 188). In addition, RNA interference can also involve small RNA (e.g., micro-RNA or miRNA) mediated gene silencing, presumably though cellular mechanisms that regulate chromatin structure and thereby prevent transcription of target gene sequences (see for example Allshire, 2002, *Science,* 297, 1818-1819; Volpe *et al.*, 2002, *Science,* 297, 1833-1837; Jenuwein, 2002, *Science,* 297, 2215-2218; and Hall *et al.*, 2002, *Science,* 297, 2232-2237). As such, siNA molecules of the invention can be used to mediate gene silencing via interaction with RNA transcripts or alternately by interaction with particular gene sequences, wherein such interaction results in gene silencing either at the transcriptional level or post-transcriptional level.

RNAi has been studied in a variety of systems. Fire *et al.*, 1998, *Nature,* 391, 806, were the first to observe RNAi in *C. elegans*. Wianny and Goetz, 1999, *Nature Cell Biol.*, 2, 70, describe RNAi mediated by dsRNA in mouse embryos. Hammond *et al.*, 2000, *Nature,* 404, 293, describe RNAi in *Drosophila* cells transfected with dsRNA. Elbashir *et al.*, 2001, *Nature,* 411, 494, describe RNAi induced by introduction of duplexes of synthetic 21-nucleotide RNAs in cultured mammalian cells including human embryonic kidney and HeLa cells. Recent work in Drosophila embryonic lysates has revealed certain requirements for siRNA length, structure, chemical composition, and sequence that are essential to mediate efficient RNAi activity. These studies have shown that 21 nucleotide siRNA duplexes are most active when containing two 2-nucleotide 3'-terminal nucleotide overhangs. Furthermore, substitution of one or both siRNA strands with 2'-deoxy or 2'-O-methyl nucleotides abolishes RNAi activity, whereas substitution of 3'-terminal siRNA nucleotides with deoxy nucleotides was shown to be tolerated.

Mismatch sequences in the center of the siRNA duplex were also shown to abolish RNAi activity. In addition, these studies also indicate that the position of the cleavage site in the target RNA is defined by the 5'-end of the siRNA guide sequence rather than the 3'-end (Elbashir *et al.*, 2001, *EMBO J.*, 20, 6877). Other studies have indicated that a 5'-phosphate on the target-complementary strand of a siRNA duplex is required for siRNA activity and that ATP is utilized to maintain the 5'-phosphate moiety on the siRNA (Nykanen *et al.*, 2001, *Cell,* 107, 309); however, siRNA molecules lacking a 5'-phosphate are active when introduced exogenously, suggesting that 5'-phosphorylation of siRNA constructs may occur *in vivo*.

Synthesis of Nucleic acid Molecules

Synthesis of nucleic acids greater than 100 nucleotides in length is difficult using automated methods, and the therapeutic cost of such molecules is prohibitive. In this invention, small nucleic acid motifs "small" refers to nucleic acid motifs no more than 100 nucleotides in length, preferably no more than 80 nucleotides in length, and most preferably no more than 50 nucleotides in length; *e.g.*, individual siNA oligonucleotide sequences or siNA sequences synthesized in tandem) are preferably used for exogenous delivery. The simple structure of these molecules increases the ability of the nucleic acid to invade targeted regions of protein and/or RNA structure. Exemplary molecules of the instant invention are chemically synthesized, and others can similarly be synthesized.

Oligonucleotides (*e.g.*, certain modified oligonucleotides or portions of oligonucleotides lacking ribonucleotides) are synthesized using protocols known in the art, for example as described in Caruthers *et al.*, 1992, *Methods in Enzymology* 211, 3-19, Thompson *et al.*, International PCT Publication No. WO 99/54459, Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677-2684, Wincott *et al.*, 1997, *Methods Mol. Bio.*, 74, 59, Brennan *et al.*, 1998, *Biotechnol Bioeng.*, 61, 33-45, and Brennan, U.S. Pat. No. 6,001,311. All of these references are incorporated herein by reference. The synthesis of oligonucleotides makes use of common nucleic acid protecting and coupling groups, such as dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μmol scale protocol with a 2.5 min coupling step for 2'-O-methylated nucleotides and a 45 sec coupling step for 2'-deoxy nucleotides or 2'-deoxy-2'-

fluoro nucleotides. **Table II** outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 μmol scale can be performed on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 μL of 0.11 M = 6.6 μmol) of 2'-O-methyl phosphoramidite and a 105-fold excess of S-ethyl tetrazole (60 μL of 0.25 M = 15 μmol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 22-fold excess (40 μL of 0.11 M = 4.4 μmol) of deoxy phosphoramidite and a 70-fold excess of S-ethyl tetrazole (40 μL of 0.25 M = 10 μmol) can be used in each coupling cycle of deoxy residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); and oxidation solution is 16.9 mM I_2, 49 mM pyridine, 9% water in THF (PERSEPTIVE™). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide, 0.05 M in acetonitrile) is used.

Deprotection of the DNA-based oligonucleotides is performed as follows: the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to –20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H2O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder.

The method of synthesis used for RNA including certain siNA molecules of the invention follows the procedure as described in Usman *et al.*, 1987, *J. Am. Chem. Soc.*, 109, 7845; Scaringe *et al.*, 1990, *Nucleic Acids Res.*, 18, 5433; and Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677-2684 Wincott *et al.*, 1997, *Methods Mol. Bio.*, 74, 59, and makes use of common nucleic acid protecting and coupling groups, such as

dimethoxytrityl at the 5'-end, and phosphoramidites at the 3'-end. In a non-limiting example, small scale syntheses are conducted on a 394 Applied Biosystems, Inc. synthesizer using a 0.2 μmol scale protocol with a 7.5 min coupling step for alkylsilyl protected nucleotides and a 2.5 min coupling step for 2'-O-methylated nucleotides. **Table II** outlines the amounts and the contact times of the reagents used in the synthesis cycle. Alternatively, syntheses at the 0.2 μmol scale can be done on a 96-well plate synthesizer, such as the instrument produced by Protogene (Palo Alto, CA) with minimal modification to the cycle. A 33-fold excess (60 μL of 0.11 M = 6.6 μmol) of 2'-O-methyl phosphoramidite and a 75-fold excess of S-ethyl tetrazole (60 μL of 0.25 M = 15 μmol) can be used in each coupling cycle of 2'-O-methyl residues relative to polymer-bound 5'-hydroxyl. A 66-fold excess (120 μL of 0.11 M = 13.2 μmol) of alkylsilyl (ribo) protected phosphoramidite and a 150-fold excess of S-ethyl tetrazole (120 μL of 0.25 M = 30 μmol) can be used in each coupling cycle of ribo residues relative to polymer-bound 5'-hydroxyl. Average coupling yields on the 394 Applied Biosystems, Inc. synthesizer, determined by colorimetric quantitation of the trityl fractions, are typically 97.5-99%. Other oligonucleotide synthesis reagents for the 394 Applied Biosystems, Inc. synthesizer include the following: detritylation solution is 3% TCA in methylene chloride (ABI); capping is performed with 16% *N*-methyl imidazole in THF (ABI) and 10% acetic anhydride/10% 2,6-lutidine in THF (ABI); oxidation solution is 16.9 mM I_2, 49 mM pyridine, 9% water in THF (PERSEPTIVE™). Burdick & Jackson Synthesis Grade acetonitrile is used directly from the reagent bottle. S-Ethyltetrazole solution (0.25 M in acetonitrile) is made up from the solid obtained from American International Chemical, Inc. Alternately, for the introduction of phosphorothioate linkages, Beaucage reagent (3H-1,2-Benzodithiol-3-one 1,1-dioxide0.05 M in acetonitrile) is used.

Deprotection of the RNA is performed using either a two-pot or one-pot protocol. For the two-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 40% aq. methylamine (1 mL) at 65 °C for 10 min. After cooling to –20 °C, the supernatant is removed from the polymer support. The support is washed three times with 1.0 mL of EtOH:MeCN:H2O/3:1:1, vortexed and the supernatant is then added to the first supernatant. The combined supernatants, containing the oligoribonucleotide, are dried to a white powder. The base deprotected oligoribonucleotide is resuspended in anhydrous

TEA/HF/NMP solution (300 µL of a solution of 1.5 mL N-methylpyrrolidinone, 750 µL TEA and 1 mL TEA•3HF to provide a 1.4 M HF concentration) and heated to 65 °C. After 1.5 h, the oligomer is quenched with 1.5 M NH_4HCO_3.

Alternatively, for the one-pot protocol, the polymer-bound trityl-on oligoribonucleotide is transferred to a 4 mL glass screw top vial and suspended in a solution of 33% ethanolic methylamine/DMSO: 1/1 (0.8 mL) at 65 °C for 15 min. The vial is brought to rt. TEA•3HF (0.1 mL) is added and the vial is heated at 65 °C for 15 min. The sample is cooled at –20 °C and then quenched with 1.5 M NH_4HCO_3.

For purification of the trityl-on oligomers, the quenched NH_4HCO_3 solution is loaded onto a C-18 containing cartridge that had been prewashed with acetonitrile followed by 50 mM TEAA. After washing the loaded cartridge with water, the RNA is detritylated with 0.5% TFA for 13 min. The cartridge is then washed again with water, salt exchanged with 1 M NaCl and washed with water again. The oligonucleotide is then eluted with 30% acetonitrile.

The average stepwise coupling yields are typically >98% (Wincott *et al.*, 1995 *Nucleic Acids Res.* 23, 2677-2684). Those of ordinary skill in the art will recognize that the scale of synthesis can be adapted to be larger or smaller than the example described above including but not limited to 96-well format.

Alternatively, the nucleic acid molecules of the present invention can be synthesized separately and joined together post-synthetically, for example, by ligation (Moore *et al.*, 1992, *Science* 256, 9923; Draper *et al.*, International PCT publication No. WO 93/23569; Shabarova *et al.*, 1991, *Nucleic Acids Research* 19, 4247; Bellon *et al.*, 1997, *Nucleosides & Nucleotides*, 16, 951; Bellon *et al.*, 1997, *Bioconjugate Chem.* 8, 204), or by hybridization following synthesis and/or deprotection.

The siNA molecules of the invention can also be synthesized via a tandem synthesis methodology as described in Example 1 herein, wherein both siNA strands are synthesized as a single contiguous oligonucleotide fragment or strand separated by a cleavable linker which is subsequently cleaved to provide separate siNA fragments or strands that hybridize and permit purification of the siNA duplex. The linker can be a polynucleotide linker or a non-nucleotide linker. The tandem synthesis of siNA as

described herein can be readily adapted to both multiwell/multiplate synthesis platforms such as 96 well or similarly larger multi-well platforms. The tandem synthesis of siNA as described herein can also be readily adapted to large scale synthesis platforms employing batch reactors, synthesis columns and the like.

A siNA molecule can also be assembled from two distinct nucleic acid strands or fragments wherein one fragment includes the sense region and the second fragment includes the antisense region of the RNA molecule.

The nucleic acid molecules of the present invention can be modified extensively to enhance stability by modification with nuclease resistant groups, for example, 2'-amino, 2'-*C*-allyl, 2'-fluoro, 2'-*O*-methyl, 2'-H (for a review see Usman and Cedergren, 1992, *TIBS* 17, 34; Usman *et al.,* 1994, *Nucleic Acids Symp. Ser.* 31, 163). siNA constructs can be purified by gel electrophoresis using general methods or can be purified by high pressure liquid chromatography (HPLC; see Wincott *et al., supra*, the totality of which is hereby incorporated herein by reference) and re-suspended in water.

In another aspect of the invention, siNA molecules of the invention are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors can be DNA plasmids or viral vectors. siNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus, retrovirus, adenovirus, or alphavirus. The recombinant vectors capable of expressing the siNA molecules can be delivered as described herein, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of siNA molecules.

Optimizing Activity of the nucleic acid molecule of the invention.

Chemically synthesizing nucleic acid molecules with modifications (base, sugar and/or phosphate) can prevent their degradation by serum ribonucleases, which can increase their potency (see *e.g.,* Eckstein *et al.,* International Publication No. WO 92/07065; Perrault *et al.,* 1990 *Nature* 344, 565; Pieken *et al.,* 1991, *Science* 253, 314; Usman and Cedergren, 1992, *Trends in Biochem. Sci.* 17, 334; Usman *et al.,* International Publication No. WO 93/15187; and Rossi *et al.,* International Publication No. WO 91/03162; Sproat, U.S. Pat. No. 5,334,711; Gold *et al.,* U.S. Pat. No. 6,300,074; and Burgin *et al., supra;* all of which are incorporated by reference herein). All of the above

references describe various chemical modifications that can be made to the base, phosphate and/or sugar moieties of the nucleic acid molecules described herein. Modifications that enhance their efficacy in cells, and removal of bases from nucleic acid molecules to shorten oligonucleotide synthesis times and reduce chemical requirements are desired.

There are several examples in the art describing sugar, base and phosphate modifications that can be introduced into nucleic acid molecules with significant enhancement in their nuclease stability and efficacy. For example, oligonucleotides are modified to enhance stability and/or enhance biological activity by modification with nuclease resistant groups, for example, 2'-amino, 2'-*C*-allyl, 2'-fluoro, 2'-*O*-methyl, 2'-O-allyl, 2'-H, nucleotide base modifications (for a review see Usman and Cedergren, 1992, *TIBS.* 17, 34; Usman *et al.,* 1994, *Nucleic Acids Symp. Ser.* 31, 163; Burgin *et al.,* 1996, *Biochemistry*, 35, 14090). Sugar modification of nucleic acid molecules have been extensively described in the art (see Eckstein *et al., International Publication* PCT No. WO 92/07065; Perrault *et al. Nature*, 1990, *344*, 565-568; Pieken *et al. Science,* 1991, *253*, 314-317; Usman and Cedergren, *Trends in Biochem. Sci.* , 1992, *17*, 334-339; Usman *et al. International Publication* PCT No. WO 93/15187; Sproat, *U.S. Pat.* No. 5,334,711 and Beigelman *et al.,* 1995, *J. Biol. Chem.,* 270, 25702; Beigelman *et al.,* International PCT publication No. WO 97/26270; Beigelman *et al.,* U.S. Pat. No. 5,716,824; Usman *et al.,* U.S. Pat. No. 5,627,053; Woolf *et al.,* International PCT Publication No. WO 98/13526; Thompson *et al.,* USSN 60/082,404 which was filed on April 20, 1998; Karpeisky *et al.,* 1998, *Tetrahedron Lett.*, 39, 1131; Earnshaw and Gait, 1998, *Biopolymers (Nucleic Acid Sciences),* 48, 39-55; Verma and Eckstein, 1998, *Annu. Rev. Biochem.,* 67, 99-134; and Burlina *et al.,* 1997, *Bioorg. Med. Chem.,* 5, 1999-2010; all of the references are hereby incorporated in their totality by reference herein). Such publications describe general methods and strategies to determine the location of incorporation of sugar, base and/or phosphate modifications and the like into nucleic acid molecules without modulating catalysis, and are incorporated by reference herein. In view of such teachings, similar modifications can be used as described herein to modify the siNA nucleic acid molecules of the instant invention so long as the ability of siNA to promote RNAi is cells is not significantly inhibited.

While chemical modification of oligonucleotide internucleotide linkages with phosphorothioate, phosphorodithioate, and/or 5'-methylphosphonate linkages improves stability, excessive modifications can cause some toxicity or decreased activity. Therefore, when designing nucleic acid molecules, the amount of these internucleotide linkages should be minimized. The reduction in the concentration of these linkages should lower toxicity, resulting in increased efficacy and higher specificity of these molecules.

Short interfering nucleic acid (siNA) molecules having chemical modifications that maintain or enhance activity are provided. Such a nucleic acid is also generally more resistant to nucleases than an unmodified nucleic acid. Accordingly, the *in vitro* and/or *in vivo* activity should not be significantly lowered. In cases in which modulation is the goal, therapeutic nucleic acid molecules delivered exogenously should optimally be stable within cells until translation of the target RNA has been modulated long enough to reduce the levels of the undesirable protein. This period of time varies between hours to days depending upon the disease state. Improvements in the chemical synthesis of RNA and DNA (Wincott *et al.*, 1995, *Nucleic Acids Res.* 23, 2677; Caruthers *et al.*, 1992, *Methods in Enzymology* 211,3-19 (incorporated by reference herein)) have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability, as described above.

In one embodiment, nucleic acid molecules of the invention include one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) G-clamp nucleotides. A G-clamp nucleotide is a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine within a duplex, see for example Lin and Matteucci, 1998, *J. Am. Chem. Soc.*, 120, 8531-8532. A single G-clamp analog substitution within an oligonucleotide can result in substantially enhanced helical thermal stability and mismatch discrimination when hybridized to complementary oligonucleotides. The inclusion of such nucleotides in nucleic acid molecules of the invention results in both enhanced affinity and specificity to nucleic acid targets, complementary sequences, or template strands. In another embodiment, nucleic acid molecules of the invention include one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) LNA "locked nucleic acid" nucleotides such as a 2', 4'-C

methylene bicyclo nucleotide (see for example Wengel *et al.,* International PCT Publication No. WO 00/66604 and WO 99/14226).

In another embodiment, the invention features conjugates and/or complexes of siNA molecules of the invention. Such conjugates and/or complexes can be used to facilitate delivery of siNA molecules into a biological system, such as a cell. The conjugates and complexes provided by the instant invention can impart therapeutic activity by transferring therapeutic compounds across cellular membranes, altering the pharmacokinetics, and/or modulating the localization of nucleic acid molecules of the invention. The present invention encompasses the design and synthesis of novel conjugates and complexes for the delivery of molecules, including, but not limited to, small molecules, lipids, phospholipids, nucleosides, nucleotides, nucleic acids, antibodies, toxins, negatively charged polymers and other polymers, for example proteins, peptides, hormones, carbohydrates, polyethylene glycols, or polyamines, across cellular membranes. In general, the transporters described are designed to be used either individually or as part of a multi-component system, with or without degradable linkers. These compounds are expected to improve delivery and/or localization of nucleic acid molecules of the invention into a number of cell types originating from different tissues, in the presence or absence of serum (see Sullenger and Cech, U.S. Pat. No. 5,854,038). Conjugates of the molecules described herein can be attached to biologically active molecules via linkers that are biodegradable, such as biodegradable nucleic acid linker molecules.

The term "biodegradable linker" as used herein, refers to a nucleic acid or non-nucleic acid linker molecule that is designed as a biodegradable linker to connect one molecule to another molecule, for example, a biologically active molecule to a siNA molecule of the invention or the sense and antisense strands of a siNA molecule of the invention. The biodegradable linker is designed such that its stability can be modulated for a particular purpose, such as delivery to a particular tissue or cell type. The stability of a nucleic acid-based biodegradable linker molecule can be modulated by using various chemistries, for example combinations of ribonucleotides, deoxyribonucleotides, and chemically-modified nucleotides, such as 2'-O-methyl, 2'-fluoro, 2'-amino, 2'-O-amino, 2'-C-allyl, 2'-O-allyl, and other 2'-modified or base modified nucleotides. The biodegradable nucleic acid linker molecule can be a dimer, trimer, tetramer or longer

nucleic acid molecule, for example, an oligonucleotide of about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length, or can comprise a single nucleotide with a phosphorus-based linkage, for example, a phosphoramidate or phosphodiester linkage. The biodegradable nucleic acid linker molecule can also comprise nucleic acid backbone, nucleic acid sugar, or nucleic acid base modifications.

The term "biodegradable" as used herein, refers to degradation in a biological system, for example enzymatic degradation or chemical degradation.

The term "biologically active molecule" as used herein, refers to compounds or molecules that are capable of eliciting or modifying a biological response in a system. Non-limiting examples of biologically active siNA molecules either alone or in combination with other molecules contemplated by the instant invention include therapeutically active molecules such as antibodies, hormones, antivirals, peptides, proteins, chemotherapeutics, small molecules, vitamins, co-factors, nucleosides, nucleotides, oligonucleotides, enzymatic nucleic acids, antisense nucleic acids, triplex forming oligonucleotides, 2,5-A chimeras, siNA, dsRNA, allozymes, aptamers, decoys and analogs thereof. Biologically active molecules of the invention also include molecules capable of modulating the pharmacokinetics and/or pharmacodynamics of other biologically active molecules, for example, lipids and polymers such as polyamines, polyamides, polyethylene glycol and other polyethers.

The term "phospholipid" as used herein, refers to a hydrophobic molecule comprising at least one phosphorus group. For example, a phospholipid can comprise a phosphorus-containing group and saturated or unsaturated alkyl group, optionally substituted with OH, COOH, oxo, amine, or substituted or unsubstituted aryl groups.

Therapeutic nucleic acid molecules (*e.g.*, siNA molecules) delivered exogenously optimally are stable within cells until reverse transcription of the RNA has been modulated long enough to reduce the levels of the RNA transcript. The nucleic acid molecules are resistant to nucleases in order to function as effective intracellular therapeutic agents. Improvements in the chemical synthesis of nucleic acid molecules described in the instant invention and in the art have expanded the ability to modify nucleic acid molecules by introducing nucleotide modifications to enhance their nuclease stability as described above.

In yet another embodiment, siNA molecules having chemical modifications that maintain or enhance enzymatic activity of proteins involved in RNAi are provided. Such nucleic acids are also generally more resistant to nucleases than unmodified nucleic acids. Thus, *in vitro* and/or *in vivo* the activity should not be significantly lowered.

Use of the nucleic acid-based molecules of the invention will lead to better treatment of the disease progression by affording the possibility of combination therapies (*e.g.*, multiple siNA molecules targeted to different genes; nucleic acid molecules coupled with known small molecule modulators; or intermittent treatment with combinations of molecules, including different motifs and/or other chemical or biological molecules). The treatment of subjects with siNA molecules can also include combinations of different types of nucleic acid molecules, such as enzymatic nucleic acid molecules (ribozymes), allozymes, antisense, 2,5-A oligoadenylate, decoys, and aptamers.

In another aspect a siNA molecule of the invention comprises one or more 5' and/or a 3'- cap structure, for example on only the sense siNA strand, the antisense siNA strand, or both siNA strands.

By "cap structure" is meant chemical modifications, which have been incorporated at either terminus of the oligonucleotide (see, for example, Adamic *et al.*, U.S. Pat. No. 5,998,203, incorporated by reference herein). These terminal modifications protect the nucleic acid molecule from exonuclease degradation, and may help in delivery and/or localization within a cell. The cap may be present at the 5'-terminus (5'-cap) or at the 3'-terminal (3'-cap) or may be present on both termini. In non-limiting examples, the 5'-cap is selected from the group consisting of glyceryl, inverted deoxy abasic residue (moiety); 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide, 4'-thio nucleotide; carbocyclic nucleotide; 1,5-anhydrohexitol nucleotide; L-nucleotides; alpha-nucleotides; modified base nucleotide; phosphorodithioate linkage; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; acyclic 3,4-dihydroxybutyl nucleotide; acyclic 3,5-dihydroxypentyl nucleotide, 3'-3'-inverted nucleotide moiety; 3'-3'-inverted abasic moiety; 3'-2'-inverted nucleotide moiety; 3'-2'-inverted abasic moiety; 1,4-butanediol phosphate; 3'-phosphoramidate; hexylphosphate; aminohexyl phosphate; 3'-phosphate; 3'-phosphorothioate; phosphorodithioate; or bridging or non-bridging methylphosphonate moiety.

In non-limiting examples, the 3'-cap is selected from the group consisting of glyceryl, inverted deoxy abasic residue (moiety), 4',5'-methylene nucleotide; 1-(beta-D-erythrofuranosyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate; 3-aminopropyl phosphate; 6-aminohexyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; *threo*-pentofuranosyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties (for more details see Beaucage and Iyer, 1993, *Tetrahedron* 49, 1925; incorporated by reference herein).

By the term "non-nucleotide" is meant any group or compound which can be incorporated into a nucleic acid chain in the place of one or more nucleotide units, including either sugar and/or phosphate substitutions, and allows the remaining bases to exhibit their enzymatic activity. The group or compound is abasic in that it does not contain a commonly recognized nucleotide base, such as adenosine, guanine, cytosine, uracil or thymine and therefore lacks a base at the 1'-position.

An "alkyl" group refers to a saturated aliphatic hydrocarbon, including straight-chain, branched-chain, and cyclic alkyl groups. Preferably, the alkyl group has 1 to 12 carbons. More preferably, it is a lower alkyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkyl group can be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2 or $N(CH_3)_2$, amino, or SH. The term also includes alkenyl groups that are unsaturated hydrocarbon groups containing at least one carbon-carbon double bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkenyl group has 1 to 12 carbons. More preferably, it is a lower alkenyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkenyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2, halogen, $N(CH_3)_2$, amino, or SH. The term "alkyl" also includes alkynyl groups that have an

unsaturated hydrocarbon group containing at least one carbon-carbon triple bond, including straight-chain, branched-chain, and cyclic groups. Preferably, the alkynyl group has 1 to 12 carbons. More preferably, it is a lower alkynyl of from 1 to 7 carbons, more preferably 1 to 4 carbons. The alkynyl group may be substituted or unsubstituted. When substituted the substituted group(s) is preferably, hydroxyl, cyano, alkoxy, =O, =S, NO_2 or $N(CH_3)_2$, amino or SH.

Such alkyl groups can also include aryl, alkylaryl, carbocyclic aryl, heterocyclic aryl, amide and ester groups. An "aryl" group refers to an aromatic group that has at least one ring having a conjugated pi electron system and includes carbocyclic aryl, heterocyclic aryl and biaryl groups, all of which may be optionally substituted. The preferred substituent(s) of aryl groups are halogen, trihalomethyl, hydroxyl, SH, OH, cyano, alkoxy, alkyl, alkenyl, alkynyl, and amino groups. An "alkylaryl" group refers to an alkyl group (as described above) covalently joined to an aryl group (as described above). Carbocyclic aryl groups are groups wherein the ring atoms on the aromatic ring are all carbon atoms. The carbon atoms are optionally substituted. Heterocyclic aryl groups are groups having from 1 to 3 heteroatoms as ring atoms in the aromatic ring and the remainder of the ring atoms are carbon atoms. Suitable heteroatoms include oxygen, sulfur, and nitrogen, and include furanyl, thienyl, pyridyl, pyrrolyl, N-lower alkyl pyrrolo, pyrimidyl, pyrazinyl, imidazolyl and the like, all optionally substituted. An "amide" refers to an -C(O)-NH-R, where R is either alkyl, aryl, alkylaryl or hydrogen. An "ester" refers to an -C(O)-OR', where R is either alkyl, aryl, alkylaryl or hydrogen.

By "nucleotide" as used herein is as recognized in the art to include natural bases (standard), and modified bases well known in the art. Such bases are generally located at the 1' position of a nucleotide sugar moiety. Nucleotides generally comprise a base, sugar and a phosphate group. The nucleotides can be unmodified or modified at the sugar, phosphate and/or base moiety, (also referred to interchangeably as nucleotide analogs, modified nucleotides, non-natural nucleotides, non-standard nucleotides and other; see, for example, Usman and McSwiggen, *supra*; Eckstein *et al.*, International PCT Publication No. WO 92/07065; Usman *et al.*, International PCT Publication No. WO 93/15187; Uhlman & Peyman, *supra*, all are hereby incorporated by reference herein). There are several examples of modified nucleic acid bases known in the art as summarized by Limbach *et al.*, 1994, *Nucleic Acids Res.* 22, 2183. Some of the non-

limiting examples of base modifications that can be introduced into nucleic acid molecules include, inosine, purine, pyridin-4-one, pyridin-2-one, phenyl, pseudouracil, 2, 4, 6-trimethoxy benzene, 3-methyl uracil, dihydrouridine, naphthyl, aminophenyl, 5-alkylcytidines (*e.g.*, 5-methylcytidine), 5-alkyluridines (*e.g.*, ribothymidine), 5-halouridine (*e.g.*, 5-bromouridine) or 6-azapyrimidines or 6-alkylpyrimidines (*e.g.* 6-methyluridine), propyne, and others (Burgin *et al.*, 1996, *Biochemistry*, 35, 14090; Uhlman & Peyman, *supra*). By "modified bases" in this aspect is meant nucleotide bases other than adenine, guanine, cytosine and uracil at 1' position or their equivalents.

In one embodiment, the invention features modified siNA molecules, with phosphate backbone modifications comprising one or more phosphorothioate, phosphorodithioate, methylphosphonate, phosphotriester, morpholino, amidate carbamate, carboxymethyl, acetamidate, polyamide, sulfonate, sulfonamide, sulfamate, formacetal, thioformacetal, and/or alkylsilyl, substitutions. For a review of oligonucleotide backbone modifications, see Hunziker and Leumann, 1995, *Nucleic Acid Analogues: Synthesis and Properties,* in *Modern Synthetic Methods,* VCH, 331-417, and Mesmaeker *et al.*, 1994, *Novel Backbone Replacements for Oligonucleotides,* in *Carbohydrate Modifications in Antisense Research,* ACS, 24-39.

By "abasic" is meant sugar moieties lacking a base or having other chemical groups in place of a base at the 1' position, see for example Adamic *et al.*, U.S. Pat. No. 5,998,203.

By "unmodified nucleoside" is meant one of the bases adenine, cytosine, guanine, thymine, or uracil joined to the 1' carbon of β-D-ribo-furanose.

By "modified nucleoside" is meant any nucleotide base which contains a modification in the chemical structure of an unmodified nucleotide base, sugar and/or phosphate. Non-limiting examples of modified nucleotides are shown by Formulae I-VII and/or other modifications described herein.

In connection with 2'-modified nucleotides as described for the present invention, by "amino" is meant 2'-NH_2 or 2'-*O*- NH_2, which can be modified or unmodified. Such modified groups are described, for example, in Eckstein *et al.*, U.S. Pat. No. 5,672,695

and Matulic-Adamic *et al.*, U.S. Pat. No. 6,248,878, which are both incorporated by reference in their entireties.

Various modifications to nucleic acid siNA structure can be made to enhance the utility of these molecules. Such modifications will enhance shelf-life, half-life *in vitro*, stability, and ease of introduction of such oligonucleotides to the target site, *e.g.*, to enhance penetration of cellular membranes, and confer the ability to recognize and bind to targeted cells.

Administration of Nucleic Acid Molecules

A siNA molecule of the invention can be adapted for use to treat any disease, infection or condition associated with gene expression, and other indications that can respond to the level of gene product in a cell or tissue, alone or in combination with other therapies. For example, a siNA molecule can comprise a delivery vehicle, including liposomes, for administration to a subject, carriers and diluents and their salts, and/or can be present in pharmaceutically acceptable formulations. Methods for the delivery of nucleic acid molecules are described in Akhtar *et al.*, 1992, *Trends Cell Bio.*, 2, 139; *Delivery Strategies for Antisense Oligonucleotide Therapeutics*, ed. Akhtar, 1995, Maurer *et al.*, 1999, *Mol. Membr. Biol.*, 16, 129-140; Hofland and Huang, 1999, *Handb. Exp. Pharmacol.*, 137, 165-192; and Lee *et al.*, 2000, *ACS Symp. Ser.*, 752, 184-192, all of which are incorporated herein by reference. Beigelman *et al.*, U.S. Pat. No. 6,395,713 and Sullivan *et al.*, PCT WO 94/02595 further describe the general methods for delivery of nucleic acid molecules. These protocols can be utilized for the delivery of virtually any nucleic acid molecule. Nucleic acid molecules can be administered to cells by a variety of methods known to those of skill in the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins (see for example Gonzalez *et al.*, 1999, *Bioconjugate Chem.*, 10, 1068-1074), biodegradable nanocapsules, and bioadhesive microspheres, or by proteinaceous vectors (O'Hare and Normand, International PCT Publication No. WO 00/53722). Alternatively, the nucleic acid/vehicle combination is locally delivered by direct injection or by use of an infusion pump. Direct injection of the nucleic acid molecules of the invention, whether subcutaneous, intramuscular, or intradermal, can take place using standard needle and syringe methodologies, or by needle-free technologies

such as those described in Conry *et al.*, 1999, *Clin. Cancer Res.*, 5, 2330-2337 and Barry *et al.*, International PCT Publication No. WO 99/31262. Many examples in the art describe CNS delivery methods of oligonucleotides by osmotic pump, (see Chun *et al.*, 1998, *Neuroscience Letters*, 257, 135-138, D'Aldin *et al.*, 1998, *Mol. Brain Research*, 55, 151-164, Dryden *et al.*, 1998, *J. Endocrinol.*, 157, 169-175, Ghirnikar *et al.*, 1998, *Neuroscience Letters*, 247, 21-24) or direct infusion (Broaddus *et al.*, 1997, *Neurosurg. Focus*, 3, article 4). Other routes of delivery include, but are not limited to oral (tablet or pill form) and/or intrathecal delivery (Gold, 1997, *Neuroscience*, 76, 1153-1158). More detailed descriptions of nucleic acid delivery and administration are provided in Sullivan *et al.*, supra, Draper *et al.*, PCT WO93/23569, Beigelman *et al.*, PCT WO99/05094, and Klimuk *et al.*, PCT WO99/04819 all of which have been incorporated by reference herein. The molecules of the instant invention can be used as pharmaceutical agents. Pharmaceutical agents prevent, modulate the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state in a subject.

In addition, the invention features the use of methods to deliver the nucleic acid molecules of the instant invention to hematopoietic cells, including monocytes and lymphocytes. These methods are described in detail by Hartmann *et al.*, 1998, *J. Phamacol. Exp. Ther.*, 285(2), 920-928; Kronenwett *et al.*, 1998, *Blood*, 91(3), 852-862; Filion and Phillips, 1997, *Biochim. Biophys. Acta.*, 1329(2), 345-356; Ma and Wei, 1996, *Leuk. Res.*, 20(11/12), 925-930; and Bongartz *et al.*, 1994, *Nucleic Acids Research*, 22(22), 4681-8. Such methods, as described above, include the use of free oligonucleitide, cationic lipid formulations, liposome formulations including pH sensitive liposomes and immunoliposomes, and bioconjugates including oligonucleotides conjugated to fusogenic peptides, for the transfection of hematopoietic cells with oligonucleotides.

Thus, the invention features a pharmaceutical composition comprising one or more nucleic acid(s) of the invention in an acceptable carrier, such as a stabilizer, buffer, and the like. The polynucleotides of the invention can be administered (*e.g.*, RNA, DNA or protein) and introduced into a subject by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be followed. The compositions of the present invention can also be formulated and used as

tablets, capsules or elixirs for oral administration, suppositories for rectal administration, sterile solutions, suspensions for injectable administration, and the other compositions known in the art.

The present invention also includes pharmaceutically acceptable formulations of the compounds described. These formulations include salts of the above compounds, *e.g.*, acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, *e.g.*, systemic administration, into a cell or subject, including for example a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation from reaching a target cell (*i.e.*, a cell to which the negatively charged nucleic acid is desirable for delivery). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms that prevent the composition or formulation from exerting its effect.

By "systemic administration" is meant *in vivo* systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes that lead to systemic absorption include, without limitation: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes exposes the siNA molecules of the invention to an accessible diseased tissue. The rate of entry of a drug into the circulation has been shown to be a function of molecular weight or size. The use of a liposome or other drug carrier comprising the compounds of the instant invention can potentially localize the drug, for example, in certain tissue types, such as the tissues of the reticular endothelial system (RES). A liposome formulation that can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach can provide enhanced delivery of the drug to target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells, such as cancer cells.

By "pharmaceutically acceptable formulation" is meant, a composition or formulation that allows for the effective distribution of the nucleic acid molecules of the instant invention in the physical location most suitable for their desired activity. Non-limiting examples of agents suitable for formulation with the nucleic acid molecules of the instant invention include: P-glycoprotein inhibitors (such as Pluronic P85), which can enhance entry of drugs into the CNS (Jolliet-Riant and Tillement, 1999, *Fundam. Clin. Pharmacol.*, 13, 16-26); biodegradable polymers, such as poly (DL-lactide-coglycolide) microspheres for sustained release delivery after intracerebral implantation (Emerich, DF *et al*, 1999, *Cell Transplant*, 8, 47-58) (Alkermes, Inc. Cambridge, MA); and loaded nanoparticles, such as those made of polybutylcyanoacrylate, which can deliver drugs across the blood brain barrier and can alter neuronal uptake mechanisms (*Prog Neuropsychopharmacol Biol Psychiatry*, 23, 941-949, 1999). Other non-limiting examples of delivery strategies for the nucleic acid molecules of the instant invention include material described in Boado *et al.*, 1998, *J. Pharm. Sci.*, 87, 1308-1315; Tyler *et al.*, 1999, *FEBS Lett.*, 421, 280-284; Pardridge *et al.*, 1995, *PNAS USA.*, 92, 5592-5596; Boado, 1995, *Adv. Drug Delivery Rev.*, 15, 73-107; Aldrian-Herrada *et al.*, 1998, *Nucleic Acids Res.*, 26, 4910-4916; and Tyler *et al.*, 1999, *PNAS USA.*, 96, 7053-7058.

The invention also features the use of the composition comprising surface-modified liposomes containing poly (ethylene glycol) lipids (PEG-modified, or long-circulating liposomes or stealth liposomes). These formulations offer a method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic *et al. Chem. Rev.* 1995, 95, 2601-2627; Ishiwata *et al., Chem. Pharm. Bull.* 1995, 43, 1005-1011). Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic *et al., Science* 1995, 267, 1275-1276; Oku *et al.*,1995, *Biochim. Biophys. Acta*, 1238, 86-90). The long-circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu *et al., J. Biol. Chem.* 1995, 42, 24864-24870; Choi *et al.*, International PCT Publication No. WO 96/10391; Ansell *et al.*, International PCT Publication No. WO 96/10390; Holland *et al.*,

International PCT Publication No. WO 96/10392). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen.

The present invention also includes compositions prepared for storage or administration that include a pharmaceutically effective amount of the desired compounds in a pharmaceutically acceptable carrier or diluent. Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co. (A.R. Gennaro edit. 1985), hereby incorporated by reference herein. For example, preservatives, stabilizers, dyes and flavoring agents can be provided. These include sodium benzoate, sorbic acid and esters of *p*-hydroxybenzoic acid. In addition, antioxidants and suspending agents can be used.

A pharmaceutically effective dose is that dose required to prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state. The pharmaceutically effective dose depends on the type of disease, the composition used, the route of administration, the type of mammal being treated, the physical characteristics of the specific mammal under consideration, concurrent medication, and other factors that those skilled in the medical arts will recognize. Generally, an amount between 0.1 mg/kg and 100 mg/kg body weight/day of active ingredients is administered dependent upon potency of the negatively charged polymer.

The nucleic acid molecules of the invention and formulations thereof can be administered orally, topically, parenterally, by inhalation or spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and/or vehicles. The term parenteral as used herein includes percutaneous, subcutaneous, intravascular (*e.g.*, intravenous), intramuscular, or intrathecal injection or infusion techniques and the like. In addition, there is provided a pharmaceutical formulation comprising a nucleic acid molecule of the invention and a pharmaceutically acceptable carrier. One or more nucleic acid molecules of the invention can be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants, and if desired other active ingredients. The pharmaceutical

compositions containing nucleic acid molecules of the invention can be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs.

Compositions intended for oral use can be prepared according to any method known to the art for the manufacture of pharmaceutical compositions and such compositions can contain one or more such sweetening agents, flavoring agents, coloring agents or preservative agents in order to provide pharmaceutically elegant and palatable preparations. Tablets contain the active ingredient in admixture with non-toxic pharmaceutically acceptable excipients that are suitable for the manufacture of tablets. These excipients can be, for example, inert diluents; such as calcium carbonate, sodium carbonate, lactose, calcium phosphate or sodium phosphate; granulating and disintegrating agents, for example, corn starch, or alginic acid; binding agents, for example starch, gelatin or acacia; and lubricating agents, for example magnesium stearate, stearic acid or talc. The tablets can be uncoated or they can be coated by known techniques. In some cases such coatings can be prepared by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained action over a longer period. For example, a time delay material such as glyceryl monosterate or glyceryl distearate can be employed.

Formulations for oral use can also be presented as hard gelatin capsules wherein the active ingredient is mixed with an inert solid diluent, for example, calcium carbonate, calcium phosphate or kaolin, or as soft gelatin capsules wherein the active ingredient is mixed with water or an oil medium, for example peanut oil, liquid paraffin or olive oil.

Aqueous suspensions contain the active materials in a mixture with excipients suitable for the manufacture of aqueous suspensions. Such excipients are suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydropropyl-methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents can be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as

polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate. The aqueous suspensions can also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.

Oily suspensions can be formulated by suspending the active ingredients in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin. The oily suspensions can contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents and flavoring agents can be added to provide palatable oral preparations. These compositions can be preserved by the addition of an anti-oxidant such as ascorbic acid

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives. Suitable dispersing or wetting agents or suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavoring and coloring agents, can also be present.

Pharmaceutical compositions of the invention can also be in the form of oil-in-water emulsions. The oily phase can be a vegetable oil or a mineral oil or mixtures of these. Suitable emulsifying agents can be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin, and esters or partial esters derived from fatty acids and hexitol, anhydrides, for example sorbitan monooleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsions can also contain sweetening and flavoring agents.

Syrups and elixirs can be formulated with sweetening agents, for example glycerol, propylene glycol, sorbitol, glucose or sucrose. Such formulations can also contain a demulcent, a preservative and flavoring and coloring agents. The pharmaceutical compositions can be in the form of a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated according to the known art using those suitable

dispersing or wetting agents and suspending agents that have been mentioned above. The sterile injectable preparation can also be a sterile injectable solution or suspension in a non-toxic parentally acceptable diluent or solvent, for example as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that can be employed are water, Ringer's solution and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose, any bland fixed oil can be employed including synthetic mono-or diglycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables.

The nucleic acid molecules of the invention can also be administered in the form of suppositories, *e.g.*, for rectal administration of the drug. These compositions can be prepared by mixing the drug with a suitable non-irritating excipient that is solid at ordinary temperatures but liquid at the rectal temperature and will therefore melt in the rectum to release the drug. Such materials include cocoa butter and polyethylene glycols.

Nucleic acid molecules of the invention can be administered parenterally in a sterile medium. The drug, depending on the vehicle and concentration used, can either be suspended or dissolved in the vehicle. Advantageously, adjuvants such as local anesthetics, preservatives and buffering agents can be dissolved in the vehicle.

Dosage levels of the order of from about 0.1 mg to about 140 mg per kilogram of body weight per day are useful in the treatment of the above-indicated conditions (about 0.5 mg to about 7 g per subject per day). The amount of active ingredient that can be combined with the carrier materials to produce a single dosage form varies depending upon the host treated and the particular mode of administration. Dosage unit forms generally contain between from about 1 mg to about 500 mg of an active ingredient.

It is understood that the specific dose level for any particular subject depends upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet, time of administration, route of administration, and rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

For administration to non-human animals, the composition can also be added to the animal feed or drinking water. It can be convenient to formulate the animal feed and

drinking water compositions so that the animal takes in a therapeutically appropriate quantity of the composition along with its diet. It can also be convenient to present the composition as a premix for addition to the feed or drinking water.

The nucleic acid molecules of the present invention can also be administered to a subject in combination with other therapeutic compounds to increase the overall therapeutic effect. The use of multiple compounds to treat an indication can increase the beneficial effects while reducing the presence of side effects.

In one embodiment, the invention comprises compositions suitable for administering nucleic acid molecules of the invention to specific cell types. For example, the asialoglycoprotein receptor (ASGPr) (Wu and Wu, 1987, *J. Biol. Chem.* 262, 4429-4432) is unique to hepatocytes and binds branched galactose-terminal glycoproteins, such as asialoorosomucoid (ASOR). In another example, the folate receptor is overexpressed in many cancer cells. Binding of such glycoproteins, synthetic glycoconjugates, or folates to the receptor takes place with an affinity that strongly depends on the degree of branching of the oligosaccharide chain, for example, triatennary structures are bound with greater affinity than biatenarry or monoatennary chains (Baenziger and Fiete, 1980, *Cell,* 22, 611-620; Connolly *et al.,* 1982, *J. Biol. Chem.,* 257, 939-945). Lee and Lee, 1987, *Glycoconjugate J.*, 4, 317-328, obtained this high specificity through the use of N-acetyl-D-galactosamine as the carbohydrate moiety, which has higher affinity for the receptor, compared to galactose. This "clustering effect" has also been described for the binding and uptake of mannosyl-terminating glycoproteins or glycoconjugates (Ponpipom *et al.,* 1981, *J. Med. Chem.,* 24, 1388-1395). The use of galactose, galactosamine, or folate based conjugates to transport exogenous compounds across cell membranes can provide a targeted delivery approach to, for example, the treatment of liver disease, cancers of the liver, or other cancers. The use of bioconjugates can also provide a reduction in the required dose of therapeutic compounds required for treatment. Furthermore, therapeutic bioavialability, pharmacodynamics, and pharmacokinetic parameters can be modulated through the use of nucleic acid bioconjugates of the invention. Non-limiting examples of such bioconjugates are described in Vargeese *et al.,* USSN 10/201,394, filed August 13, 2001; and Matulic-Adamic *et al.,* USSN 60/362,016, filed March 6, 2002.

Examples:

The following are non-limiting examples showing the selection, isolation, synthesis and activity of nucleic acids of the instant invention.

Example 1: Tandem synthesis of siNA constructs

Exemplary siNA molecules of the invention are synthesized in tandem using a cleavable linker, for example, a succinyl-based linker. Tandem synthesis as described herein is followed by a one-step purification process that provides RNAi molecules in high yield. This approach is highly amenable to siNA synthesis in support of high throughput RNAi screening, and can be readily adapted to multi-column or multi-well synthesis platforms.

After completing a tandem synthesis of a siNA oligo and its complement in which the 5'-terminal dimethoxytrityl (5'-O-DMT) group remains intact (trityl on synthesis), the oligonucleotides are deprotected as described above. Following deprotection, the siNA sequence strands are allowed to spontaneously hybridize. This hybridization yields a duplex in which one strand has retained the 5'-O-DMT group while the complementary strand comprises a terminal 5'-hydroxyl. The newly formed duplex behaves as a single molecule during routine solid-phase extraction purification (Trityl-On purification) even though only one molecule has a dimethoxytrityl group. Because the strands form a stable duplex, this dimethoxytrityl group (or an equivalent group, such as other trityl groups or other hydrophobic moieties) is all that is required to purify the pair of oligos, for example, by using a C18 cartridge.

Standard phosphoramidite synthesis chemistry is used up to the point of introducing a tandem linker, such as an inverted deoxy abasic succinate or glyceryl succinate linker (see **Figure 1**) or an equivalent cleavable linker. A non-limiting example of linker coupling conditions that can be used includes a hindered base such as diisopropylethylamine (DIPA) and/or DMAP in the presence of an activator reagent such as Bromotripyrrolidinophosphoniumhexaflurorophosphate (PyBrOP). After the linker is coupled, standard synthesis chemistry is utilized to complete synthesis of the second sequence leaving the terminal the 5'-O-DMT intact. Following synthesis, the resulting oligonucleotide is deprotected according to the procedures described herein and quenched with a suitable buffer, for example with 50mM NaOAc or 1.5M $NH_4H_2CO_3$.

Purification of the siNA duplex can be readily accomplished using solid phase extraction, for example using a Waters C18 SepPak 1g cartridge conditioned with 1 column volume (CV) of acetonitrile, 2 CV H2O, and 2 CV 50mM NaOAc. The sample is loaded and then washed with 1 CV H2O or 50mM NaOAc. Failure sequences are eluted with 1 CV 14% ACN (Aqueous with 50mM NaOAc and 50mM NaCl). The column is then washed, for example with 1 CV H2O followed by on-column detritylation, for example by passing 1 CV of 1% aqueous trifluoroacetic acid (TFA) over the column, then adding a second CV of 1% aqueous TFA to the column and allowing to stand for approximately 10 minutes. The remaining TFA solution is removed and the column washed with H20 followed by 1 CV 1M NaCl and additional H2O. The siNA duplex product is then eluted, for example, using 1 CV 20% aqueous CAN.

Figure 2 provides an example of MALDI-TOV mass spectrometry analysis of a purified siNA construct in which each peak corresponds to the calculated mass of an individual siNA strand of the siNA duplex. The same purified siNA provides three peaks when analyzed by capillary gel electrophoresis (CGE), one peak presumably corresponding to the duplex siNA, and two peaks presumably corresponding to the separate siNA sequence strands. Ion exchange HPLC analysis of the same siNA contract only shows a single peak. Testing of the purified siNA construct using a luciferase reporter assay described below demonstrated the same RNAi activity compared to siNA constructs generated from separately synthesized oligonucleotide sequence strands.

Example 2: Serum stability of chemically modified siNA constructs

Chemical modifications were introduced into siNA constructs to determine the stability of these constructs compared to native siNA oligonucleotides (containing two thymidine nucleotide overhangs) in human serum. An investigation of the serum stability of RNA duplexes revealed that siNA constructs consisting of all RNA nucleotides containing two thymidine nucleotide overhangs have a half-life in serum of 15 seconds, whereas chemically modified siNA constructs remained stable in serum for 1 to 3 days depending on the extent of modification (see Figure 3). RNAi stability tests were performed by internally labeling one strand (strand 1) of siNA and duplexing with 1.5 X the concentration of the complementary siNA strand (strand 2) (to insure all labeled material was in duplex form). Duplexed siNA constructs were then tested for stability by

incubating at a final concentration of 2μM siNA (strand 2 concentration) in 90% mouse or human serum for time-points of 30sec, 1min, 5min, 30min, 90min, 4hrs 10min, 16hrs 24min, and 49hrs. Time points were run on a 15% denaturing polyacrylamide gels and analyzed on a phosphoimager.

Internal labeling was performed via kinase reactions with polynucleotide kinase (PNK) and ^{32}P-γ-ATP, with addition of radiolabeled phosphate at nucleotide 13 of strand 2, counting in from the 3' side. Ligation of the remaining 8-mer fragments with T4 RNA ligase resulted in the full length, 21-mer, strand 2. Duplexing of RNAi was done by adding appropriate concentrations of the siNA oligonucleotides and heating to 95° C for 5minutes followed by slow cooling to room temperature. Reactions were performed by adding 100% serum to the siNA duplexes and incubating at 37° C, then removing aliquots at desired time-points. Results of this study are summarized in **Figure 3**. As shown in the Figure 3, chemically modified siNA molecules (e.g., SEQ ID NOs: 412/413, 412/414, 412/415, 412/416, and 412/418) have significantly increased serum stability compared to an siNA construct having all ribonucleotides except a 3'-terminal dithymidine (TT) modification (e.g., SEQ ID NOs: 419/420).

Example 3: Identification of potential siNA target sites in any RNA sequence

The sequence of an RNA target of interest, such as a viral or human mRNA transcript, is screened for target sites, for example by using a computer folding algorithm. In a non-limiting example, the sequence of a gene or RNA gene transcript derived from a database, such as Genbank, is used to generate siNA targets having complementarity to the target. Such sequences can be obtained from a database, or can be determined experimentally as known in the art. Target sites that are known, for example, those target sites determined to be effective target sites based on studies with other nucleic acid molecules, for example ribozymes or antisense, or those targets known to be associated with a disease or condition such as those sites containing mutations or deletions, can be used to design siNA molecules targeting those sites. Various parameters can be used to determine which sites are the most suitable target sites within the target RNA sequence. These parameters include but are not limited to secondary or tertiary RNA structure, the nucleotide base composition of the target sequence, the degree of homology between various regions of the target sequence, or the relative position of the target sequence

within the RNA transcript. Based on these determinations, any number of target sites within the RNA transcript can be chosen to screen siNA molecules for efficacy, for example by using *in vitro* RNA cleavage assays, cell culture, or animal models. In a non-limiting example, anywhere from 1 to 1000 target sites are chosen within the transcript based on the size of the siNA construct to be used. High throughput screening assays can be developed for screening siNA molecules using methods known in the art, such as with multi-well or multi-plate assays or combinatorial/siNA library screening assays to determine efficient reduction in target gene expression.

Example 4: Selection of siNA molecule target sites in a RNA

The following non-limiting steps can be used to carry out the selection of siNAs targeting a given gene sequence or transcript.

The target sequence is parsed in silico into a list of all fragments or subsequences of a particular length, for example 23 nucleotide fragments, contained within the target sequence. This step is typically carried out using a custom Perl script, but commercial sequence analysis programs such as Oligo, MacVector, or the GCG Wisconsin Package can be employed as well.

In some instances the siNAs correspond to more than one target sequence; such would be the case for example in targeting different transcripts of the same gene, targeting different transcripts of more than one gene, or for targeting both the human gene and an animal homolog. In this case, a subsequence list of a particular length is generated for each of the targets, and then the lists are compared to find matching sequences in each list. The subsequences are then ranked according to the number of target sequences that contain the given subsequence; the goal is to find subsequences that are present in most or all of the target sequences. Alternately, the ranking can identify subsequences that are unique to a target sequence, such as a mutant target sequence. Such an approach would enable the use of siNA to target specifically the mutant sequence and not effect the expression of the normal sequence.

In some instances the siNA subsequences are absent in one or more sequences while present in the desired target sequence; such would be the case if the siNA targets a gene with a paralogous family member that is to remain untargeted. As in case 2 above, a

subsequence list of a particular length is generated for each of the targets, and then the lists are compared to find sequences that are present in the target gene but are absent in the untargeted paralog.

The ranked siNA subsequences can be further analyzed and ranked according to GC content. A preference can be given to sites containing 30-70% GC, with a further preference to sites containing 40-60% GC.

The ranked siNA subsequences can be further analyzed and ranked according to self-folding and internal hairpins. Weaker internal folds are preferred; strong hairpin structures are to be avoided.

The ranked siNA subsequences can be further analyzed and ranked according to whether they have runs of GGG or CCC in the sequence. GGG (or even more Gs) in either strand can make oligonucleotide synthesis problematic and can potentially interfere with RNAi activity, so it is avoided other appropriately suitable sequences are available. CCC is searched in the target strand because that will place GGG in the antisense strand.

The ranked siNA subsequences can be further analyzed and ranked according to whether they have the dinucleotide UU (uridine dinucleotide) on the 3'-end of the sequence, and/or AA on the 5'-end of the sequence (to yield 3' UU on the antisense sequence). These sequences allow one to design siNA molecules with terminal TT thymidine dinucleotides.

Four or five target sites are chosen from the ranked list of subsequences as described above. For example, in subsequences having 23 nucleotides, the right 21 nucleotides of each chosen 23-mer subsequence are then designed and synthesized for the upper (sense) strand of the siNA duplex, while the reverse complement of the left 21 nucleotides of each chosen 23-mer subsequence are then designed and synthesized for the lower (antisense) strand of the siNA duplex (see Tables I). If terminal TT residues are desired for the sequence (as described in paragraph 7), then the two 3' terminal nucleotides of both the sense and antisense strands are replaced by TT prior to synthesizing the oligos.

The siNA molecules are screened in an in vitro, cell culture or animal model system to identify the most active siNA molecule or the most preferred target site within the target RNA sequence.

In an alternate approach, a pool of siNA constructs specific to a target sequence is used to screen for target sites in cells expressing target RNA, such as human HeLa cells. The general strategy used in this approach is shown in **Figure 21**. A non-limiting example of such a pool is a pool comprising sequences having antisense sequences complementary to the target RNA sequence and sense sequences complementary to the antisense sequences. Cells (e.g., HeLa cells) expressing the target gene are transfected with the pool of siNA constructs and cells that demonstrate a phenotype associated with gene silencing are sorted. The pool of siNA constructs can be chemically modified as described herein and synthesized, for example, in a high throughput manner. The siNA from cells demonstrating a positive phenotypic change (e.g., decreased target mRNA levels or target protein expression), are identified, for example by positional analysis within the assay, and are used to determine the most suitable target site(s) within the target RNA sequence based upon the complementary sequence to the corresponding siNA antisense strand identified in the assay.

Example 5: RNAi activity of chemically modified siNA constructs

Short interfering nucleic acid (siNA) is emerging as a powerful tool for gene regulation. All-ribose siNA duplexes activate the RNAi pathway but have limited utility as therapeutic compounds due to their nuclease sensitivity and short half-life in serum, as shown in Example 2 above. To develop nuclease-resistant siNA constructs for *in vivo* applications, siNAs that target luciferase mRNA and contain stabilizing chemical modifications were tested for activity in HeLa cells. The sequences for the siNA oligonucleotide sequences used in this study are shown in **Table I**. Modifications included phosphorothioate linkages (P=S), 2'–*O*–methyl nucleotides, or 2'–fluoro (F) nucleotides in one or both siNA strands and various 3'-end stabilization chemistries, including 3'-glyceryl, 3'-inverted abasic, 3'-inverted Thymidine, and/or Thymidine. The RNAi activity of chemically stabilized siNA constructs was compared with the RNAi activity of control siNA constructs consisting of all ribonucleotides at every position except the 3'-terminus which comprised two thymidine nucleotide overhangs. Active

siNA molecules containing stabilizing modifications such as described herein should prove useful for *in vivo* applications, given their enhanced nuclease-resistance.

A luciferase reporter system was utilized to test RNAi activity of chemically modified siNA constructs compared to siNA constructs consisting of all RNA nucleotides containing two thymidine nucleotide overhangs. Sense and antisense siNA strands (20 uM each) were annealed by incubation in buffer (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) for 1 min. at 90°C followed by 1 hour at 37°C. Plasmids encoding firefly luciferase (pGL2) and renilla luciferase (pRLSV40) were purchased from Promega Biotech.

HeLa S3 cells were grown at 37°C in DMEM with 5% FBS and seeded at 15,300 cells in 100 ul media per well of a 96-well plate 24 hours prior to transfection. For transfection, 4 ul Lipofectamine 2000 (Life Technologies) was added to 96 ul OPTI-MEM, vortexed and incubated at room temperature for 5 minutes. The 100 ul diluted lipid was then added to a microtiter tube containing 5 ul pGL2 (200ng/ul), 5 ul pRLSV40 (8 ng/ul) 6 ul siNA (25 nM or 10 nM final), and 84 ul OPTI-MEM, vortexed briefly and incubated at room temperature for 20 minutes. The transfection mix was then mixed briefly and 50 ul was added to each of three wells that contained HeLa S3 cells in 100 ul media. Cells were incubated for 20 hours after transfection and analyzed for luciferase expression using the Dual luciferase assay according to the manufacturer's instructions (Promega Biotech). The results of this study are summarized in **Figures 4-16**. The sequences of the siNA strands used in this study are shown in Table I and are referred to by RPI# in the figures. Normalized luciferase activity is reported as the ratio of firefly luciferase activity to renilla luciferase activity in the same sample. Error bars represent standard deviation of triplicate transfections. As shown in **Figures 4-16**, the RNAi activity of chemically modified constructs is often comparable to that of unmodified control siNA constructs, which consist of all ribonucleotides at every position except the 3'-terminus which comprises two thymidine nucleotide overhangs. In some instances, the RNAi activity of the chemically modified constructs is greater than the unmodified control siNA construct consisting of all ribonucleotides..

For example, **Figure 4** shows results obtained from a screen using phosphorothioate modified siNA constructs. The RPI 27654/27659 construct contains phosphorothioate

substitutions for every pyrimidine nucleotide in both sequences, the RPI 27657/27662 construct contains 5 terminal 3'-phosphorothioate substitutions in each strand, the RPI 27649/27658 construct contains all phosphorothioate substitutions only in the antisense strand, whereas the RPI 27649/27660 and RPI 27649/27661 constructs have unmodified sense strands and varying degrees of phosphorothioate substitutions in the antisense strand. All of these constructs show significant RNAi activity when compared to a scrambled siNA conrol construct (27651/27652).

Figure 5 shows results obtained from a screen using phosphorothioate (RPI 28253/28255 and RPI 28254/28256) and universal base substitutions (RPI 28257/28259 and RPI 28258/28260) compared to the same controls described above, these modifications show equivalent or better RNAi activity when compared to the unmodified control siNA construct.

Figure 6 shows results obtained from a screen using 2'-O-methyl modified siNA constructs in which the sense strand contains either 10 (RPI 28244/27650) or 5 (RPI 28245/27650) 2'-O-methyl substitutions, both with comparable activity to the unmodified control siNA construct.

Figure 7 shows results obtained from a screen using 2'-O-methyl or 2'-deoxy-2'-fluoro modified siNA constructs compared to a control construct consisting of all ribonucleotides at every position except the 3'-terminus which comprises two thymidine nucleotide overhangs.

Figure 8 compares a siNA construct containing six phosphorothioate substitutions in each strand (RPI 28460/28461), where 5 phosphorothioates are present at the 3' end and a single phosphorothioate is present at the 5' end of each strand. This motif shows very similar activity to the control siNA construct consisting of all ribonucleotides at every position except the 3'-terminus, which comprises two thymidine nucleotide overhangs.

Figure 9 compares a siNA construct synthesized by the method of the invention described in Example 1, wherein an inverted deoxyabasic succinate linker was used to generate a siNA having a 3'-inverted deoxyabasic cap on the antisense strand of the siNA. This construct shows improved activity compared to the control siNA construct

consisting of all ribonucleotides at every position except the 3'-terminus which comprises two thymidine nucleotide overhangs.

Figure 10 shows the results of an RNAi activity screen of chemically modifed siNA constructs including 3'-glyceryl modified siNA constructs compared to an all RNA control siNA construct using a luciferase reporter system. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by RPI number (sense strand/antisense strand). Sequences correspoding to these RPI numbers are shown in Table I. As shown in the Figure, the 3'-terminal modified siNA constructs retain significant RNAi activity compared to the unmodified control siNA (siGL2) construct.

Figure 11 shows the results of an RNAi activity screen of chemically modifed siNA constructs. The screen compared various combinations of sense strand chemical modifications and antisense strand chemical modifications. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by RPI number (sense strand/antisense strand). Sequences correspoding to these RPI numbers are shown in Table I. As shown in the figure, the chemically modified RPI 30063/30430, RPI 30433/30430, and RPI 30063/30224 constructs retain significant RNAi activity compared to the unmodified control siNA construct. It should be noted that RPI 30433/30430 is a siNA construct having no ribonucleotides which retains significant RNAi activity compared to the unmodified control siGL2 construct in vitro, therefore, this construct is expected to have both similar RNAi activity and improved stability in vivo compared to siNA constructs having ribonucleotides.

Figure 12 shows the results of an RNAi activity screen of chemically modifed siNA constructs. The screen compared various combinations of sense strand chemical

modifications and antisense strand chemical modifications. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by RPI number (sense strand/antisense strand). Sequences correspoding to these RPI numbers are shown in Table I. As shown in the figure, the chemically modified RPI 30063/30224 and RPI 30063/30430 constructs retain significant RNAi activity compared to the control siNA (siGL2) construct. In addition, the antisense strand alone (RPI 30430) and an inverted control (RPI 30227/30229), having matched chemistry to RPI (30063/30224) were compared to the siNA duplexes described above. The antisense strand (RPI 30430) alone provides far less inhibition compared to the siNA duplexes using this sequence.

Figure 13 shows the results of an RNAi activity screen of chemically modifed siNA constructs. The screen compared various combinations of sense strand chemical modifications and antisense strand chemical modifications. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by RPI number (sense strand/antisense strand). Sequences correspoding to these RPI numbers are shown in Table I. In addition, an inverted control (RPI 30226/30229, having matched chemistry to RPI 30222/30224) was compared to the siNA duplexes described above. As shown in the figure, the chemically modified RPI 28251/30430, RPI 28251/30224, and RPI 30222/30224 constructs retain significant RNAi activity compared to the control siNA construct, and the chemically modified RPI 28251/30430 construct demonstrates improved activity compared to the control siNA (siGL2) construct.

Figure 14 shows the results of an RNAi activity screen of chemically modifed siNA constructs including various 3'-terminal modified siNA constructs compared to an all RNA control siNA construct using a luciferase reporter system. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and

10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by RPI number (sense strand/antisense strand). Sequences correspoding to these RPI numbers are shown in Table I. As shown in the figure, the chemically modified RPI 30222/30546, 30222/30224, 30222/30551, 30222/30557 and 30222/30558 constructs retain significant RNAi activity compared to the control siNA construct.

Figure 15 shows the results of an RNAi activity screen of chemically modifed siNA constructs. The screen compared various combinations of sense strand chemistries compared to a fixed antisense strand chemistry. These chemically modified siNAs were compared in the luciferase assay described herein at 1 nM and 10nM concentration using an all RNA siNA control (siGL2) having 3'-terminal dithymidine (TT) and its corresponding inverted control (Inv siGL2). The background level of luciferase expression in the HeLa cells is designated by the "cells" column. Sense and antisense strands of chemically modified siNA constructs are shown by RPI number (sense strand/antisense strand). Sequences correspoding to these RPI numbers are shown in Table I. As shown in the figure, the chemically modified RPI 30063/30430, 30434/30430, and 30435/30430 constructs all demonstrate greater activity compared to the control siNA (siGL2) construct.

Example 6: RNAi activity titration

A titration assay was performed to determine the lower range of siNA concentration required for RNAi activity both in a control siNA construct consisting of all RNA nucleotides containing two thymidine nucleotide overhangs and a chemically modified siNA construct comprising five phosphorothioate internucleotide linkages in both the sense and antisense strands. The assay was performed as described above, however, the siNA constructs were diluted to final concentrations between 2.5 nM and 0.025 nM. Results are shown in **Figure 16**. As shown in **Figure 16**, the chemically modified siNA construct shows a very similar concentration dependent RNAi activity profile to the control siNA construct when compared to an inverted siNA sequence control.

Example 7: siNA design

siNA target sites were chosen by analyzing sequences of the target RNA and optionally prioritizing the target sites on the basis of folding (structure of any given sequence analyzed to determine siNA accessibility to the target), by using a library of siNA molecules as described in Example 4, or alternately by using an *in vitro* siNA system as described in Example 9 herein. siNA molecules were designed that could bind each target and are optionally individually analyzed by computer folding to assess whether the siNA molecule can interact with the target sequence. Varying the length of the siNA molecules can be chosen to optimize activity. Generally, a sufficient number of complementary nucleotide bases are chosen to bind to, or otherwise interact with, the target RNA, but the degree of complementarity can be modulated to accommodate siNA duplexes or varying length or base composition. By using such methodologies, siNA molecules can be designed to target sites within any known RNA sequence, for example those RNA sequences corresponding to the any gene transcript.

Chemically modified siNA constructs are designed to provide nuclease stability for systemic administration in vivo and/or improved pharmacokinetic, localization, and delivery properties while preserving the ability to mediate RNAi activity. Chemical modifications as described herein are introduced synthetically using synthetic methods described herein and those generally known in the art. The synthetic siNA constructs are then assayed for nuclease stability in serum and/or cellular/tissue extracts (e.g. liver extracts). The synthetic siNA constructs are also tested in parallel for RNAi activity using an appropriate assay, such as a luciferase reporter assay as described herein or another suitable assay that can quantity RNAi activity. Synthetic siNA constructs that possess both nuclease stability and RNAi activity can be further modified and re-evaluated in stability and activity assays. The chemical modifications of the stabilized active siNA constructs can then be applied to any siNA sequence targeting any chosen RNA and used, for example, in target screening assays to pick lead siNA compounds for therapeutic development (see for example **Figure 27**).

Example 8: Chemical Synthesis and Purification of siNA

siNA molecules can be designed to interact with various sites in the RNA message, for example, target sequences within the RNA sequences described herein. The sequence

of one strand of the siNA molecule(s) is complementary to the target site sequences described above. The siNA molecules can be chemically synthesized using methods described herein. Inactive siNA molecules that are used as control sequences can be synthesized by scrambling the sequence of the siNA molecules such that it is not complementary to the target sequence. Generally, siNA constructs can by synthesized using solid phase oligonucleotide synthesis methods as described herein (see for example Usman *et al.*, US Patent Nos. 5,804,683; 5,831,071; 5,998,203; 6,117,657; 6,353,098; 6,362,323; 6,437,117; 6,469,158; Scaringe *et al.*, US Patent Nos. 6,111,086; 6,008,400; 6,111,086 all incorporated by reference herein in their entirety).

In a non-limiting example, RNA oligonucleotides are synthesized in a stepwise fashion using the phosphoramidite chemistry as is known in the art. Standard phosphoramidite chemistry involves the use of nucleosides comprising any of 5'-O-dimethoxytrityl, 2'-O-tert-butyldimethylsilyl, 3'-O-2-Cyanoethyl N, N-diisopropylphosphoroamidite groups, and exocyclic amine protecting groups (e.g. N6-benzoyl adenosine, N4 acetyl cytidine, and N2-isobutyryl guanosine). Alternately, 2′-O-Silyl Ethers can be used in conjunction with acid-labile 2′-O-orthoester protecting groups in the synthesis of RNA as described by Scaringe *supra*. Differing 2' chemistries can require different protecting groups, for example 2'-deoxy-2'-amino nucleosides can utilize N-phthaloyl protection as described by Usman *et al.*, US Patent 5,631,360, incorporated by reference herein in its entirety).

During solid phase synthesis, each nucleotide is added sequentially (3′- to 5′-direction) to the solid support-bound oligonucleotide. The first nucleoside at the 3′-end of the chain is covalently attached to a solid support (e.g., controlled pore glass or polystyrene) using various linkers. The nucleotide precursor, a ribonucleoside phosphoramidite, and activator are combined resulting in the coupling of the second nucleoside phosphoramidite onto the 5′-end of the first nucleoside. The support is then washed and any unreacted 5′-hydroxyl groups are capped with a capping reagent such as acetic anhydride to yield inactive 5′-acetyl moieties. The trivalent phosphorus linkage is then oxidized to a more stable phosphate linkage. At the end of the nucleotide addition cycle, the 5′-O-protecting group is cleaved under suitable conditions (e.g., acidic conditions for trityl-based groups and Fluoride for silyl-based groups). The cycle is repeated for each subsequent nucleotide.

Modification of synthesis conditions can be used to optimize coupling efficiency, for example by using differing coupling times, differing reagent/phosphoramidite concentrations, differing contact times, differing solid supports and solid support linker chemistries depending on the particular chemical composition of the siNA to be synthesized. Deprotection and purification of the siNA can be performed as is generally described in Usman et al., US 5,831,071, US 6,353,098, US 6,437,117, and Bellon et al., US 6,054,576, US 6,162,909, US 6,303,773, incorporated by reference herein in their entirety or Scaringe *supra*,. Additionally, deprotection conditions can be modified to provide the best possible yield and purity of siNA constructs. For example, applicant has observed that oligonucleotides comprising 2'-deoxy-2'-fluoro nucleotides can degrade under inappropriate deprotection conditions. Such oligonucleotides are deprotected using aqueous methylamine at about 35°C for 30 minutes. If the 2'-deoxy-2'-fluoro containing oligonucleotide also comprises ribonucleotides, after deprotection with aqueous methylamine at about 35°C for 30 minutes, TEA-HF is added and the reaction maintained at about 65°C for an additional 15 minutes.

Example 9: RNAi *in vitro* assay to assess siNA activity

An in vitro assay that recapitulates RNAi in a cell free system is used to evaluate siNA constructs specific to target RNA. The assay comprises the system described by Tuschl *et al.*, 1999, *Genes and Development*, 13, 3191-3197 and Zamore *et al.*, 2000, *Cell*, 101, 25-33 adapted for use with target RNA. A Drosophila extract derived from syncytial blastoderm is used to reconstitute RNAi activity *in vitro*. Target RNA is generated via *in vitro* transcription from an appropriate plasmid using T7 RNA polymerase or via chemical synthesis as described herein. Sense and antisense siNA strands (for example 20 uM each) are annealed by incubation in buffer (such as 100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) for 1 min. at 90°C followed by 1 hour at 37°C , then diluted in lysis buffer (for example 100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2mM magnesium acetate). Annealing can be monitored by gel electrophoresis on an agarose gel in TBE buffer and stained with ethidium bromide. The Drosophila lysate is prepared using zero to two-hour-old embryos from Oregon R flies collected on yeasted molasses agar that are dechorionated and lysed. The lysate is centrifuged and the supernatant isolated. The assay comprises a reaction mixture containing 50% lysate [vol/vol], RNA (10-50 pM final concentration), and 10%

[vol/vol] lysis buffer containing siNA (10 nM final concentration). The reaction mixture also contains 10 mM creatine phosphate, 10 ug.ml creatine phosphokinase, 100 um GTP, 100 uM UTP, 100 uM CTP, 500 uM ATP, 5 mM DTT, 0.1 U/uL RNasin (Promega), and 100 uM of each amino acid. The final concentration of potassium acetate is adjusted to 100 mM. The reactions are pre-assembled on ice and preincubated at 25° C for 10 minutes before adding RNA, then incubated at 25° C for an additional 60 minutes. Reactions are quenched with 4 volumes of 1.25 x Passive Lysis Buffer (Promega). Target RNA cleavage is assayed by RT-PCR analysis or other methods known in the art and are compared to control reactions in which siNA is omitted from the reaction.

Alternately, internally-labeled target RNA for the assay is prepared by *in vitro* transcription in the presence of [alpha-^{32}P] CTP, passed over a G 50 Sephadex column by spin chromatography and used as target RNA without further purification. Optionally, target RNA is 5'-^{32}P-end labeled using T4 polynucleotide kinase enzyme. Assays are performed as described above and target RNA and the specific RNA cleavage products generated by RNAi are visualized on an autoradiograph of a gel. The percentage of cleavage is determined by Phosphor Imager® quantitation of bands representing intact control RNA or RNA from control reactions without siNA and the cleavage products generated by the assay.

In one embodiment, this assay is used to determine target sites the RNA target for siNA mediated RNAi cleavage, wherein a plurality of siNA constructs are screened for RNAi mediated cleavage of the RNA target, for example, by analyzing the assay reaction by electrophoresis of labeled target RNA, or by northern blotting, as well as by other methodology well known in the art.

Example 10: Nucleic acid inhibition of target RNA *in vivo*

siNA molecules targeted to the target RNA are designed and synthesized as described above. These nucleic acid molecules can be tested for cleavage activity *in vivo*, for example, using the following procedure.

Two formats are used to test the efficacy of siNAs targeting a particular gene transcipt. First, the reagents are tested on target expressing cells (e.g., HeLa), to

determine the extent of RNA and protein inhibition. siNA reagents are selected against the RNA target. RNA inhibition is measured after delivery of these reagents by a suitable transfection agent to cells. Relative amounts of target RNA are measured versus actin using real-time PCR monitoring of amplification (*eg.*, ABI 7700 Taqman®). A comparison is made to a mixture of oligonucleotide sequences made to unrelated targets or to a randomized siNA control with the same overall length and chemistry, but with randomly substituted nucleotides at each position. Primary and secondary lead reagents are chosen for the target and optimization performed. After an optimal transfection agent concentration is chosen, a RNA time-course of inhibition is performed with the lead siNA molecule. In addition, a cell-plating format can be used to determine RNA inhibition.

Delivery of siNA to Cells

Cells (e.g., HeLa) are seeded, for example, at $1x10^5$ cells per well of a six-well dish in EGM-2 (BioWhittaker) the day before transfection. siNA (final concentration, for example 20nM) and cationic lipid (e.g., final concentration 2μg/ml) are complexed in EGM basal media (Biowhittaker) at 37°C for 30 mins in polystyrene tubes. Following vortexing, the complexed siNA is added to each well and incubated for the times indicated. For initial optimization experiments, cells are seeded, for example, at $1x10^3$ in 96 well plates and siNA complex added as described. Efficiency of delivery of siNA to cells is determined using a fluorescent siNA complexed with lipid. Cells in 6-well dishes are incubated with siNA for 24 hours, rinsed with PBS and fixed in 2% paraformaldehyde for 15 minutes at room temperature. Uptake of siNA is visualized using a fluorescent microscope.

Taqman and Lightcycler quantification of mRNA

Total RNA is prepared from cells following siNA delivery, for example, using Qiagen RNA purification kits for 6-well or Rneasy extraction kits for 96-well assays. For Taqman analysis, dual-labeled probes are synthesized with the reporter dye, FAM or JOE, covalently linked at the 5'-end and the quencher dye TAMRA conjugated to the 3'-end. One-step RT-PCR amplifications are performed on, for example, an ABI PRISM 7700 Sequence Detector using 50 μl reactions consisting of 10 μl total RNA, 100 nM forward primer, 900 nM reverse primer, 100 nM probe, 1X TaqMan PCR reaction buffer (PE-

Applied Biosystems), 5.5 mM $MgCl_2$, 300 μM each dATP, dCTP, dGTP, and dTTP, 10U RNase Inhibitor (Promega), 1.25U AmpliTaq Gold (PE-Applied Biosystems) and 10U M-MLV Reverse Transcriptase (Promega). The thermal cycling conditions can consist of 30 min at 48°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Quantitation of mRNA levels is determined relative to standards generated from serially diluted total cellular RNA (300, 100, 33, 11 ng/rxn) and normalizing to ß-actin or GAPDH mRNA in parallel TaqMan reactions. For each gene of interest an upper and lower primer and a fluorescently labeled probe are designed. Real time incorporation of SYBR Green I dye into a specific PCR product can be measured in glass capillary tubes using a lightcyler. A standard curve is generated for each primer pair using control cRNA. Values are represented as relative expression to GAPDH in each sample.

Western blotting

Nuclear extracts can be prepared using a standard micro preparation technique (see for example Andrews and Faller, 1991, *Nucleic Acids Research,* 19, 2499). Protein extracts from supernatants are prepared, for example using TCA precipitation. An equal volume of 20% TCA is added to the cell supernatant, incubated on ice for 1 hour and pelleted by centrifugation for 5 minutes. Pellets are washed in acetone, dried and resuspended in water. Cellular protein extracts are run on a 10% Bis-Tris NuPage (nuclear extracts) or 4-12% Tris-Glycine (supernatant extracts) polyacrylamide gel and transferred onto nitro-cellulose membranes. Non-specific binding can be blocked by incubation, for example, with 5% non-fat milk for 1 hour followed by primary antibody for 16 hour at 4°C. Following washes, the secondary antibody is applied, for example (1:10,000 dilution) for 1 hour at room temperature and the signal detected with SuperSignal reagent (Pierce).

Example 11: Animal Models

Various animal models can be used to screen siNA constructs *in vivo* as are known in the art, for example those animal models that are used to evaluate other nucleic acid technologies such as enzymatic nucleic acid molecules (ribozymes) and/or antisense. Such animal models are used to test the efficacy of siNA molecules described herein. In a non-limiting example, siNA molecules that are designed as anti-angiogenic agents can be screened using animal models. There are several animal models available in which to

test the anti-angiogenesis effect of nucleic acids of the present invention, such as siNA, directed against genes associated with angiogenesis and/or metastais, such as VEGFR (e.g., VEGFR1, VEGFR2, and VEGFR3) genes. Typically a corneal model has been used to study angiogenesis in rat and rabbit, since recruitment of vessels can easily be followed in this normally avascular tissue (Pandey *et al.*, 1995 *Science* 268: 567-569). In these models, a small Teflon or Hydron disk pretreated with an angiogenesis factor (e.g. bFGF or VEGF) is inserted into a pocket surgically created in the cornea. Angiogenesis is monitored 3 to 5 days later. siNA molecules directed against VEGFR mRNAs would be delivered in the disk as well, or dropwise to the eye over the time course of the experiment. In another eye model, hypoxia has been shown to cause both increased expression of VEGF and neovascularization in the retina (Pierce *et al.*, 1995 *Proc. Natl. Acad. Sci.* USA. 92: 905-909; Shweiki *et al.*, 1992 *J. Clin. Invest.* 91: 2235-2243).

Several animal models exist for screening of anti-angiogenic agents. These include corneal vessel formation following corneal injury (Burger *et al.*, 1985 *Cornea* 4: 35-41; Lepri, *et al.*, 1994 *J. Ocular Pharmacol.* 10: 273-280; Ormerod *et al.*, 1990 *Am. J. Pathol.* 137: 1243-1252) or intracorneal growth factor implant (Grant *et al.*, 1993 *Diabetologia* 36: 282-291; Pandey *et al.* 1995 *supra;* Zieche *et al.*, 1992 *Lab. Invest.* 67: 711-715), vessel growth into Matrigel matrix containing growth factors (Passaniti *et al.*, 1992 *supra*), female reproductive organ neovascularization following hormonal manipulation (Shweiki *et al.*, 1993 *Clin. Invest.* 91: 2235-2243), several models involving inhibition of tumor growth in highly vascularized solid tumors (O'Reilly *et al.*, 1994 *Cell* 79: 315-328; Senger *et al.*, 1993 *Cancer and Metas. Rev.* 12: 303-324; Takahasi *et al.*, 1994 *Cancer Res.* 54: 4233-4237; Kim *et al.*, 1993 *supra*), and transient hypoxia-induced neovascularization in the mouse retina (Pierce *et al.*, 1995 *Proc. Natl. Acad. Sci.* USA. 92: 905-909).gene

The cornea model, described in Pandey et al. *supra*, is the most common and well characterized anti-angiogenic agent efficacy screening model. This model involves an avascular tissue into which vessels are recruited by a stimulating agent (growth factor, thermal or alkalai burn, endotoxin). The corneal model utilizes the intrastromal corneal implantation of a Teflon pellet soaked in a VEGF-Hydron solution to recruit blood vessels toward the pellet, which can be quantitated using standard microscopic and image analysis techniques. To evaluate their anti-angiogenic efficacy, siNA molecules are

applied topically to the eye or bound within Hydron on the Teflon pellet itself. This avascular cornea as well as the Matrigel model (described below) provide for low background assays. While the corneal model has been performed extensively in the rabbit, studies in the rat have also been conducted.

The mouse model (Passaniti et al., *supra*) is a non-tissue model which utilizes Matrigel, an extract of basement membrane (Kleinman et al., 1986) or Millipore® filter disk, which can be impregnated with growth factors and anti-angiogenic agents in a liquid form prior to injection. Upon subcutaneous administration at body temperature, the Matrigel or Millipore® filter disk forms a solid implant. VEGF embedded in the Matrigel or Millipore® filter disk is used to recruit vessels within the matrix of the Matrigel or Millipore® filter disk which can be processed histologically for endothelial cell specific vWF (factor VIII antigen) immunohistochemistry, Trichrome-Masson stain, or hemoglobin content. Like the cornea, the Matrigel or Millipore® filter disk are avascular; however, it is not tissue. In the Matrigel or Millipore® filter disk model, siNA molecules are administered within the matrix of the Matrigel or Millipore® filter disk to test their anti-angiogenic efficacy. Thus, delivery issues in this model, as with delivery of siNA molecules by Hydron- coated Teflon pellets in the rat cornea model, may be less problematic due to the homogeneous presence of the siNA within the respective matrix.

The Lewis lung carcinoma and B-16 murine melanoma models are well accepted models of primary and metastatic cancer and are used for initial screening of anti-cancer agents. These murine models are not dependent upon the use of immunodeficient mice, are relatively inexpensive, and minimize housing concerns. Both the Lewis lung and B-16 melanoma models involve subcutaneous implantation of approximately 10^6 tumor cells from metastatically aggressive tumor cell lines (Lewis lung lines 3LL or D122, LLc-LN7; B-16-BL6 melanoma) in C57BL/6J mice. Alternatively, the Lewis lung model can be produced by the surgical implantation of tumor spheres (approximately 0.8 mm in diameter). Metastasis also may be modeled by injecting the tumor cells directly intraveneously. In the Lewis lung model, microscopic metastases can be observed approximately 14 days following implantation with quantifiable macroscopic metastatic tumors developing within 21-25 days. The B-16 melanoma exhibits a similar time course with tumor neovascularization beginning 4 days following implantation. Since both

primary and metastatic tumors exist in these models after 21-25 days in the same animal, multiple measurements can be taken as indices of efficacy. Primary tumor volume and growth latency as well as the number of micro- and macroscopic metastatic lung foci or number of animals exhibiting metastases can be quantitated. The percent increase in lifespan can also be measured. Thus, these models would provide suitable primary efficacy assays for screening systemically administered siNA molecules and siNA formulations.

In the Lewis lung and B-16 melanoma models, systemic pharmacotherapy with a wide variety of agents usually begins 1-7 days following tumor implantation/inoculation with either continuous or multiple administration regimens. Concurrent pharmacokinetic studies can be performed to determine whether sufficient tissue levels of siNA can be achieved for pharmacodynamic effect to be expected. Furthermore, primary tumors and secondary lung metastases can be removed and subjected to a variety of *in vitro* studies (*i.e.* target RNA reduction).

In utilizing these models to assess siNA activity, VEGFR1, VEGFR2, and/or VEGFR3 protein levels can be measured clinically or experimentally by FACS analysis. VEGFR1, VEGFR2, and/or VEGFR3 encoded mRNA levels can be assessed by Northern analysis, RNase-protection, primer extension analysis and/or quantitative RT-PCR. siNA molecules that block VEGFR1, VEGFR2, and/or VEGFR3 protein encoding mRNAs and therefore result in decreased levels of VEGFR1, VEGFR2, and/or VEGFR3 activity by more than 20% *in vitro* can be identified using the techniques described herein.

Example 12: siNA-mediated inhibition of angiogenesis *in vivo*

The purpose of this study was to assess the anti-angiogenic activity of siNA targeted against VEGFR1, using the rat cornea model of VEGF induced angiogenesis discussed in Example 11 above). The siNA molecules shown in Figure 23 have matched inverted controls which are inactive since they are not able to interact with the RNA target. The siNA molecules and VEGF were co-delivered using the filter disk method. Nitrocellulose filter disks (Millipore®) of 0.057 diameter were immersed in appropriate solutions and were surgically implanted in rat cornea as described by Pandey *et al., supra.*

The stimulus for angiogenesis in this study was the treatment of the filter disk with 30 μM VEGF which is implanted within the cornea's stroma. This dose yields reproducible neovascularization stemming from the pericorneal vascular plexus growing toward the disk in a dose-response study 5 days following implant. Filter disks treated only with the vehicle for VEGF show no angiogenic response. The siNA were co-adminstered with VEGF on a disk in three different siNA concentrations. One concern with the simultaneous administration is that the siNA would not be able to inhibit angiogenesis since VEGF receptors can be stimulated. However, Applicant has observed that in low VEGF doses, the neovascular response reverts to normal suggesting that the VEGF stimulus is essential for maintaining the angiogenic response. Blocking the production of VEGF receptors using simultaneous administration of anti-VEGF-R mRNA siNA could attenuate the normal neovascularization induced by the filter disk treated with VEGF.

Materials and Methods:

Test Compounds and Controls

R&D Systems VEGF, carrier free at 75 μM in 82 mM Tris-Cl, pH 6.9

siNA, 1.67 μG/μL, SITE 2340 (RPI 29695/29699) sense/antisense

siNA, 1.67 μG/μL, INVERTED CONTROL FOR SITE 2340 (RPI 29983/29984) sense/antisense

siNA 1.67 μg/μL, Site 2340 (RPI 30196/30416) sense/antisense

Animals

Harlan Sprague-Dawley Rats, Approximately 225-250g

45 males, 5 animals per group.

Husbandry

Animals are housed in groups of two. Feed, water, temperature and humidity are determined according to Pharmacology Testing Facility performance standards (SOP's) which are in accordance with the 1996 Guide for the Care and Use of Laboratory Animals

(NRC). Animals are acclimated to the facility for at least 7 days prior to experimentation. During this time, animals are observed for overall health and sentinels are bled for baseline serology.

Experimental Groups

Each solution (VEGF and siNAs) was prepared as a 1X solution for final concentrations shown in the experimental groups described in **Table III.**

siNA Annealing Conditions

siNA sense and antisense strands are annealed for 1 minute in H_2O at 1.67mg/mL/strand followed by a 1 hour incubation at 37°C producing 3.34 mg/mL of duplexed siNA. For the 20μg/eye treatment, 6 μLs of the 3.34 mg/mL duplex is injected into the eye (see below). The 3.34 mg/mL duplex siNA can then be serially diluted for dose response assays.

Preparation of VEGF Filter Disk

For corneal implantation, 0.57 mm diameter nitrocellulose disks, prepared from 0.45 μm pore diameter nitrocellulose filter membranes (Millipore Corporation), were soaked for 30 min in 1 μL of 75 μM VEGF in 82 mM Tris·HCl (pH 6.9) in covered petri dishes on ice. Filter disks soaked only with the vehicle for VEGF (83 mM Tris-Cl pH 6.9) elicit no angiogenic response.

Corneal surgery

The rat corneal model used in this study was a modified from Koch *et al. Supra* and Pandey *et al., supra.* Briefly, corneas were irrigated with 0.5% povidone iodine solution followed by normal saline and two drops of 2% lidocaine. Under a dissecting microscope (Leica MZ-6), a stromal pocket was created and a presoaked filter disk (see above) was inserted into the pocket such that its edge was 1 mm from the corneal limbus.

Intraconjunctival injection of test solutions

Immediately after disk insertion, the tip of a 40-50 μm OD injector (constructed in our laboratory) was inserted within the conjunctival tissue 1 mm away from the edge of the corneal limbus that was directly adjacent to the VEGF-soaked filter disk. Six hundred nanoliters of test solution (siNA, inverted control or sterile water vehicle) were dispensed at a rate of 1.2 μL/min using a syringe pump (Kd Scientific). The injector was then removed, serially rinsed in 70% ethanol and sterile water and immersed in sterile water between each injection. Once the test solution was injected, closure of the eyelid was maintained using microaneurism clips until the animal began to recover gross motor activity. Following treatment, animals were warmed on a heating pad at 37°C.

Quantitation of angiogenic response

Five days after disk implantation, animals were euthanized following administration of 0.4 mg/kg atropine and corneas were digitally imaged. The neovascular surface area (NSA, expressed in pixels) was measured *postmortem* from blood-filled corneal vessels using computerized morphometry (Image Pro Plus, Media Cybernetics, v2.0). The individual mean NSA was determined in triplicate from three regions of identical size in the area of maximal neovascularization between the filter disk and the limbus. The number of pixels corresponding to the blood-filled corneal vessels in these regions was summated to produce an index of NSA. A group mean NSA was then calculated. Data from each treatment group were normalized to VEGF/siNA vehicle-treated control NSA and finally expressed as percent inhibition of VEGF-induced angiogenesis.

Statistics

After determining the normality of treatment group means, group mean percent inhibition of VEGF-induced angiogenesis was subjected to a one-way analysis of variance. This was followed by two post-hoc tests for significance including Dunnett's (comparison to VEGF control) and Tukey-Kramer (all other group mean comparisons) at alpha = 0.05. Statistical analyses were performed using JMP v.3.1.6 (SAS Institute).

Results are graphically represented in **Figure 23**. As shown in **Figure 23**, VEGFr1 site 4229 active siNA (RPI 29695/29699) at three concentrations were effective at inhibiting angiogenesis compared to the inverted siNA control (RPI 2983/29984) and the VEGF control. A chemically modified version of the VEGFr1 site 4229 active siNA comprising a sense strand having 2'-deoxy-2'-fluoro pyrimidines and ribo purines with 5' and 3' terminal inverted deoxyabasic residues and an antisense strand having having 2'-deoxy-2'-fluoro pyrimidines and ribo purines with a terminal 3'-phosphorothioate internucleotide linkage (RPI 30196/30416), showed similar inhibition. This result shows siNA molecules having different chemically modified composition, such as the modifications described herein, are capable of significantly inhibiting angiogenesis *in vivo*.

Example 13: Inhibition of HBV using siNA Molecules of the Invention

Transfection of HepG2 Cells with psHBV-1 and siNA

The human hepatocellular carcinoma cell line Hep G2 was grown in Dulbecco's modified Eagle media supplemented with 10% fetal calf serum, 2 mM glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 25 mM Hepes, 100 units penicillin, and 100 μg/ml streptomycin. To generate a replication competent cDNA, prior to transfection the HBV genomic sequences are excised from the bacterial plasmid sequence contained in the psHBV-1 vector. Other methodsknown in the art can be used to generate a replication competent cDNA. This was done with an EcoRI and Hind III restriction digest. Following completion of the digest, a ligation was performed under dilute conditions (20 μg/ml) to favor intermolecular ligation. The total ligation mixture was then concentrated using Qiagen spin columns.

siNA Activity Screen and Dose Response Assay

Transfection of the human hepatocellular carcinoma cell line, Hep G2, with replication-competent HBV DNA results in the expression of HBV proteins and the production of virions. To test the efficacy of siNAs targeted against HBV RNA, several siNA duplexes targeting different sites within HBV pregenomic RNA were co-transfected with HBV genomic DNA once at 25 nM with lipid at 12.5 ug/ml into Hep G2 cells, and the subsequent levels of secreted HBV surface antigen (HBsAg) were analyzed by ELISA

(see **Figure 24**). Inverted sequence duplexes were used as negative controls. Subsequently, dose response studies were performed in which the siNA duplexes were co-transfected with HBV genomic DNA at 0.5, 5, 10 and 25 nM with lipid at 12.5 ug/ml into Hep G2 cells, and the subsequent levels of secreted HBV surface antigen (HBsAg) were analyzed by ELISA (see **Figure 25**).

Analysis of HBsAg Levels Following siNA Treatment

To determine siNA activity, HbsAg levels were measured following transfection with siNA. Immulon 4 (Dynax) microtiter wells were coated overnight at 4° C with anti-HBsAg Mab (Biostride B88-95-31ad,ay) at 1 μg/ml in Carbonate Buffer (Na2CO3 15 mM, NaHCO3 35 mM, pH 9.5). The wells were then washed 4x with PBST (PBS, 0.05% Tween® 20) and blocked for 1 hr at 37° C with PBST, 1% BSA. Following washing as above, the wells were dried at 37° C for 30 min. Biotinylated goat ant-HBsAg (Accurate YVS1807) was diluted 1:1000 in PBST and incubated in the wells for 1 hr. at 37° C. The wells were washed 4x with PBST. Streptavidin/Alkaline Phosphatase Conjugate (Pierce 21324) was diluted to 250 ng/ml in PBST, and incubated in the wells for 1 hr. at 37° C. After washing as above, p-nitrophenyl phosphate substrate (Pierce 37620) was added to the wells, which were then incubated for 1 hour at 37° C. The optical density at 405 nm was then determined. Results of the HBV screen study are summarized in **Figure 24**, whereas the results of a dose response assay using lead siNA constructs targeting sites 262 and 1580 of the HBV pregenomic RNA are shown in **Figure 25**. As shown in **Figure 25**, the siNA constructs targeting sites 262 and 1580 of HBV RNA provides significant dose response inhibition of viral replication/activity when compared to inverted siNA controls.

Comparison of different chemically stabilized siNA motifs targeting HBV RNA site 1580

Two different siNA stabilization chemistries were compared in a dose response HBsAg assay using inverted matched chemistry controls. The "Stab7/8" (**Table IV**) constructs comprise a sense strand having 2'-deoxy-2'-fluoro pyrimidine nucleotides and 2'-deoxy purine nucleotides with 5' and 3' terminal inverted deoxyabasic residues and an antisense strand having 2'-deoxy-2'-fluoro pyrimidine nucleotides and 2'-O-methyl purine nucleotides with a terminal 3' phosphorothioate linkage. The "Stab7/11 (**Table**

IV) constructs comprise a sense strand having 2'-deoxy-2'-fluoro pyrimidine nucleotides and 2'-deoxy purine nucleotides with 5' and 3' terminal inverted deoxyabasic residues and an antisense strand having 2'-deoxy-2'-fluoro pyrimidine nucleotides and 2'-deoxy purine nucleotides with a terminal 3' phosphorothioate linkage (see for example **Table I**). As shown in **Figure 26**, the chemically stabilized siNA constructs both show significant inhibition of HBV antigen in a dose dependent manner compared to matched inverted contols.

Time course evaluation of different chemically stabilized siNA motifs targeting HBV RNA site 1580

Four different siNA constructs having different stabilization chemistries were compared to an unstabilized siRNA construct in a dose response time course HBsAg assay, the results of which are shown in Figures 28-31. The different constructs were compared to an unstabilized ribonucleotide control siRNA construct (RPI#30287/30298) at different concentrations (5nM, 10 nM, 25 nM, 50 nM, and 100 nM) over the course of nine days. Activity based on HBsAg levels was determined at day 3, day 6, and day 9. The "Stab 4/5" (**Table IV**) constructs comprise a sense strand (RPI#30355) having 2'-deoxy-2'-fluoro pyrimidine nucleotides and purine ribonucleotides with 5' and 3' terminal inverted deoxyabasic residues and an antisense strand (RPI#30366) having 2'-deoxy-2'-fluoro pyrimidine nucleotides and purine ribonucleotides with a terminal 3' phosphorothioate linkage (data shown in **Figure 28**). The "Stab7/8" (**Table IV**) constructs comprise a sense strand (RPI#30612) having 2'-deoxy-2'-fluoro pyrimidine nucleotides and 2'-deoxy purine nucleotides with 5' and 3' terminal inverted deoxyabasic residues and an antisense strand (RPI#30620) having 2'-deoxy-2'-fluoro pyrimidine nucleotides and 2'-O-methyl purine nucleotides with a terminal 3' phosphorothioate linkage (data shown in **Figure 29**). The "Stab7/11 (**Table IV**) constructs comprise a sense (RPI#30612) strand having 2'-deoxy-2'-fluoro pyrimidine nucleotides and 2'-deoxy purine nucleotides with 5' and 3' terminal inverted deoxyabasic residues and an antisense strand (RPI#31175) having 2'-deoxy-2'-fluoro pyrimidine nucleotides and 2'-deoxy purine nucleotides with a terminal 3' phosphorothioate linkage (data shown in **Figure 30**). The "Stab9/10 (**Table IV**) constructs comprise a sense (RPI#31335) strand having ribonucleotides with 5' and 3' terminal inverted deoxyabasic residues and an antisense strand (RPI#31337) having ribonucleotides with a terminal 3' phosphorothioate linkage

(data shown in **Figure 31**). As shown in **Figures 28-31**, the chemically stabilized siNA constructs all show significantly greater inhibition of HBV antigen in a dose dependent manner over the time course experiment compared to the unstabilized siRNA construct.

Example 14: Inhibition of HCV using siNA Molecules of the Invention

siNA Inhibition of a chimeric HCV/Poliovirus in HeLa Cells

Inhibition of a chimeric HCV/Poliovirus was investigated using 21 nucleotide siNA duplexes in HeLa cells. Seven siNA constructs were designed that target three regions in the highly conserved 5' untranslated region (UTR) of HCV RNA. The siNAs were screened in two cell culture systems dependent upon the 5'-UTR of HCV; one requires translation of an HCV/luciferase gene, while the other involves replication of a chimeric HCV/poliovirus (PV) (see Blatt *et al.,* USSN 09/740,332, filed December 18, 2000, incorporated by reference herein). Two siNAs (29579/29586; 29578/29585) targeting the same region (shifted by one nucleotide) are active in both systems (see **Figure 32**) as compared with inverse control siNA (29593/29600). For example, a >85% reduction in HCVPV replication was observed in siNA-treated cells compared to an inverse siNA control (**Figure 32**) with an IC50 = ~2.5 nM (**Figure 33**). To develop nuclease-resistant siNA for in vivo applications, siNAs can be modified to contain stabilizing chemical modifications. Such modifications include phosphorothioate linkages (P=S), 2'–*O*–methyl nucleotides, 2'–fluoro (F) nucleotides, 2'-deoxy nucleotides, universal base nucleotides, 5' and/or 3' end modifications and a variety of other nucleotide and non-nucleotide modifications, in one or both siNA strands. Several of these constructs were tested in the HCV/poliovirus chimera system, demonstrating significant reduction in viral replication (**Figures 34-37**). siNA constructs shown in **Figures 34-37** are referred to by RPI#s that are cross referenced to **Table III**, which shows the sequence and chemical modifications of the constructs. siNA activity is compared to relevant controls (untreated cells, scrambled/inactive control sequences, or transfection controls). As shown in the Figures, siNA constructs of the invention provide potent inhibition of HCV RNA in the HCV/poliovirus chimera system. As such, siNA constructs, inlcuding chemically modified, nuclease resistant siNA molecules, represent an important class of therapeutic agents for treating chronic HCV infection.

siNA Inhibition of a HCV RNA expression in a HCV replicon system

In addition, a HCV replicaon system was used to test the efficacy of siNAs targeting HCV RNA. The reagents are tested in cell culture using Huh7 cells (see for example Randall *et al.*, 2003, *PNAS USA*, 100, 235-240) to determine the extent of RNA and protein inhibition. siNA were selected against the HCV target as described herein. RNA inhibition was measured after delivery of these reagents by a suitable transfection agent to Huh7 cells. Relative amounts of target RNA are measured versus actin using real-time PCR monitoring of amplification (*eg.*, ABI 7700 Taqman®). A comparison is made to a mixture of oligonucleotide sequences designed to target unrelated targets or to a randomized siNA control with the same overall length and chemistry, but with randomly substituted nucleotides at each position. Primary and secondary lead reagents were chosen for the target and optimization performed. After an optimal transfection agent concentration is chosen, a RNA time-course of inhibition is performed with the lead siNA molecule. In addition, a cell-plating format can be used to determine RNA inhibition. A non-limiting example of a multiple target screen to assay siNA mediated inhibition of HCV RNA is shown in **Figure 38**. siNA reagents (Table I) were transfected at 25 nM into Huh7 cells and HCV RNA quantitated compared to untreated cells ("cells" column in the figure) and cells transfected with lipofectamine ("LFA2K" column in the figure). As shown in the Figure, several siNA constructs show significant inhibition of HCV RNA expression in the Huh7 replicon system.

Example 15: Target Discovery in Mammalian Cells using siNA molecules

In a non-limiting example, compositions and methods of the invention are used to discover genes involved in a process of interest within mammalian cells, such as cell growth, proliferation, apoptosis, morphology, angiogenesis, differentiation, migration, viral multiplication, drug resistance, signal transduction, cell cycle regulation, or temperature sensitivity or other process. First, a randomized siNA library is generated. These constructs are inserted into a vector capable of expressing a siNA from the library inside mammalian cells. Alternately, a pool of synthetic siNA molecules is generated.

Reporter System

In order to discover genes playing a role in the expression of certain proteins, such as proteins involved in a cellular process described herein, a readily assayable reporter system is constructed in which a reporter molecule is co-expressed when a particular protein of interest is expressed. The reporter system consists of a plasmid construct bearing a gene coding for a reporter gene, such as Green Fluorescent Protein (GFP) or other reporter proteins known and readily available in the art. The promoter region of the GFP gene is replaced by a portion of a promoter for the protein of interest sufficient to direct efficient transcription of the GFP gene. The plasmid can also contain a drug resistance gene, such as neomycin resistance, in order to select cells containing the plasmid.

Host Cell Lines for Target Discovery

A cell line is selected as host for target discovery. The cell line is preferably known to express the protein of interest, such that upstream genes controlling the expression of the protein can be identified when modulated by a siNA construct expressed therein. The cells preferably retain protein expression characteristics in culture. The reporter plasmid is transfected into cells, for example, using a cationic lipid formulation. Following transfection, the cells are subjected to limiting dilution cloning, for example, under selection by 600 μg/mL Geneticin. Cells retaining the plasmid survive the Geneticin treatment and form colonies derived from single surviving cells. The resulting clonal cell lines are screened by flow cytometry for the capacity to upregulate GFP production. Treating the cells with, for example, sterilized M9 bacterial medium in which *Pseudomonas aeruginosa* had been cultured (Pseudomonas conditioned medium, PCM) is used to induce the promoter. The PCM is supplemented with phorbol myristate acetate (PMA). A clonal cell line highly responsive to promoter induction is selected as the reporter line for subsequent studies.

siNA Library Construction

A siNA library was constructed with oligonucletides containing hairpin siNA constructs having randomized antisense regions and self complementary sense regions. The library is generated synthesizing siNA constructs having randomized sequence. Alternately, the siNA libraries are constructed as described in Usman *et al.*, USSN 60/402,996 (incorporated by reference herein) Oligo sequence 5' and 3' of the siNA

contains restriction endonuclease cleavage sites for cloning. The 3' trailing sequence forms a stem-loop for priming DNA polymerase extension to form a hairpin structure. The hairpin DNA construct is melted at 90°C allowing DNA polymerase to generate a dsDNA construct. The double-stranded siNA library is cloned into, for example, a U6+27 transcription unit located in the 5' LTR region of a retroviral vector containing the human nerve growth factor receptor (hNGFr) reporter gene. Positioning the U6+27/siNA transcription unit in the 5' LTR results in a duplication of the transcription unit when the vector integrates into the host cell genome. As a result, the siNA is transcribed by RNA polymerase III from U6+27 and by RNA polymerase II activity directed by the 5' LTR. The siNA library is packaged into retroviral particles that are used to infect and transduce clonal cells selected above. Assays of the hNGFr reporter are used to indicate the percentage of cells that incorporated the siNA construct. By randomized region is meant a region of completely random sequence and/or partially random sequence. By completely random sequence is meant a sequence wherein theoretically there is equal representation of A, T, G and C nucleotides or modified derivatives thereof, at each position in the sequence. By partially random sequence is meant a sequence wherein there is an unequal representation of A, T, G and C nucleotides or modified derivatives thereof, at each position in the sequence. A partially random sequence can therefore have one or more positions of complete randomness and one or more positions with defined nucleotides.

Enriching for Non-responders to Induction

Sorting of siNA library-containing cells is performed to enrich for cells that produce less reporter GFP after treatment with the promoter inducers PCM and PMA. Lower GFP production cancan be due to RNAi activity against genes involved in the activation of the mucin promoter. Alternatively, siNA can directly target the mucin/GFP transcript resulting in reduced GFP expression.

Cells are seeded at a certain density, such as 1 x 10^6 per 150 cm^2 style cell culture flasks and grown in the appropriate cell culture medium with fetal bovine serum. After 72 hours, the cell culture medium is replaced with serum-free medium. After 24 hours of serum deprivation, the cells are treated with serum-containing medium supplemented with PCM (to 40%) and PMA (to 50 nM) to induced GFP production. After 20 to 22

hours, cells are monitored for GFP level on, for example, a FACStar Plus cell sorter. Sorting is performed if ≥90% of siNA library cells from an unsorted control sample were induced to produce GFP above background levels. Two cell fractions are collected in each round of sorting. Following the appropriate round of sorting, the M1 fraction is selected to generate a database of siNA molecules present in the sorted cells.

Recovery of siNA Sequence from Sorted Cells

Genomic DNA is obtained from sorted siNA library cells by standard methods. Nested polymerase chain reaction (PCR) primers that hybridized to the retroviral vector 5' and 3' of the siNA are used to recover and amplify the siNA sequences from the particular clone of library cell DNA. The PCR product is ligated into a bacterial cloning vector. The recovered siNA library in plasmid form can be used to generate a database of siNA sequences. For example, the library is cloned into *E. coli*. DNA is prepared by plasmid isolation from bacterial colonies or by direct colony PCR and siNA sequence is determined. A second method can use the siNA library to transfect cloned cells. Clonal lines of stably transfected cells are established and induced with, for example, PCM and PMA. Those lines which fail to respond to GFP induction are probed by PCR for single siNA integration events. The unique siNA sequences obtained by both methods are added to a Target Sequence Tag (TST) database.

Bioinformatics

The antisense region sequences of the isolated siNA constructs are compared to public and private gene data banks. Gene matches are compiled according to perfect and imperfect matches. Potential gene targets are categorized by the number of different siNA sequences matching each gene. Genes with more than one perfect siNA match are selected for Target Validation studies.

Validation of the Target Gene

To validate a target as a regulator of protein expression, siNA reagents are designed to the target gene cDNA sequence from Genbank. The siNA reagents are complexed with a cationic lipid formulation prior to administration to cloned cells at appropriate concentrations (e.g. 5-50 nM or less). Cells are treated with siNA reagents, for example from 72 to 96 hours. Before the termination of siNA treatment, PCM (to 40 %) and PMA

(to 50 nM), for example, are added to induce the promoter. After twenty hours of induction the cells are harvested and assayed for phenotypic and molecular parameters. Reduced GFP expression in siNA treated cells (measured by flow cytometry) is taken as evidence for validation of the target gene. Knockdown of target RNA in siNA treated cells can correlate with reduced endogenous RNA and reduced GFP RNA to complete validation of the target.

Example 16: Screening siNA constructs for improved pharmacokinetics

In a non-limiting example, siNA constructs are screened in vivo for improved pharmacokinetic properties compared to all RNA or unmodified siNA constructs. Chemical modifications are introduced into the siNA construct based on educated design parameters (e.g. introducing 2'-mofications, base modifications, backbone modifications, terminal cap modifications, or covalently attached conjugates etc). The modified construct in tested in an appropriate system (e.g human serum for nuclease resistance, shown, or an animal model for PK/delivery parameters). In parallel, the siNA construct is tested for RNAi activity, for example in a cell culture system such as a luciferase reporter assay). Lead siNA constructs are then identified which possess a particular characteristic while maintaining RNAi activity, and can be further modified and assayed once again. This same approach can be used to identify siNA-conjugate molecules with improved pharmacokinetic profiles, delivery, localized delivery, cellular uptake, and RNAi activity.

Example 17: Indications

The siNA molecules of the invention can be used to treat a variety of diseases and conditions through modulation of gene expression. Using the methods described herein, chemically modified siNA molecules can be designed to modulate the expression any number of target genes, including but not limited to genes associated with cancer, metabolic diseases, infectious diseases such as viral, bacterial or fungal infections, neurologic diseases, musculoskeletal diseases, diseases of the immune system, diseases associated with signaling pathways and cellular messengers, and diseases associated with transport systems including molecular pumps and channels.

Non-limiting examples of various viral genes that can be targeted using siNA molecules of the invention include Hepatitis C Virus (HCV, for example Genbank

Accession Nos: D11168, D50483.1, L38318 and S82227), Hepatitis B Virus (HBV, for example GenBank Accession No. AF100308.1), Human Immunodeficiency Virus type 1 (HIV-1, for example GenBank Accession No. U51188), Human Immunodeficiency Virus type 2 (HIV-2, for example GenBank Accession No. X60667), West Nile Virus (WNV for example GenBank accession No. NC_001563), cytomegalovirus (CMV for example GenBank Accession No. NC_001347), respiratory syncytial virus (RSV for example GenBank Accession No. NC_001781), influenza virus (for example example GenBank Accession No. AF037412, rhinovirus (for example, GenBank accession numbers: D00239, X02316, X01087, L24917, M16248, K02121, X01087), papillomavirus (for example GenBank Accession No. NC_001353), Herpes Simplex Virus (HSV for example GenBank Accession No. NC_001345), and other viruses such as HTLV (for example GenBank Accession No. AJ430458). Due to the high sequence variability of many viral genomes, selection of siNA molecules for broad therapeutic applications would likely involve the conserved regions of the viral genome. Nonlimiting examples of conserved regions of the viral genomes include but are not limited to 5'-Non Coding Regions (NCR), 3'- Non Coding Regions (NCR) LTR regions and/or internal ribosome entry sites (IRES). siNA molecules designed against conserved regions of various viral genomes will enable efficient inhibition of viral replication in diverse patient populations and may ensure the effectiveness of the siNA molecules against viral quasi species which evolve due to mutations in the non-conserved regions of the viral genome.

Non-limiting examples of human genes that can be targeted using siNA molecules of the invention using methods described herein include any human RNA sequence, for example those commonly referred to by Genbank Accession Number. These RNA sequences can be used to design siNA molecules that inhibit gene expression and therefore abrogate diseases, conditions, or infections associated with expression of those genes. Such non-limiting examples of human genes that can be targeted using siNA molecules of the invention include VEGFr (VEGFR1 for example GenBank Accession No. XM_067723, VEGFR2 for example GenBank Accession No. AF063658), HER1, HER2, HER3, and HER4 (for example Genbank Accession Nos: NM_005228, NM_004448, NM_001982, and NM_005235 respectively), telomerase (TERT, for example GenBank Accession No. NM_003219), telomerase RNA (for example GenBank

Accession No. U86046), NFkappaB, Rel-A (for example GenBank Accession No. NM_005228), NOGO (for example GenBank Accession No. AB020693), NOGOr (for example GenBank Accession No. XM_015620), RAS (for example GenBank Accession No. NM_004283), RAF (for example GenBank Accession No. XM_033884), CD20 (for example GenBank Accession No. X07203), METAP2 (for example GenBank Accession No. NM_003219), CLCA1 (for example GenBank Accession No. NM_001285), phospholamban (for example GenBank Accession No. NM_002667), PTP1B (for example GenBank Accession No. M31724), PCNA (for example GenBank Accession No. NM_002592.1), PKC-alpha (for example GenBank Accession No. NM_002737) and others. The genes described herein are provided as non-limiting examples of genes that can be targeted using siNA molecules of the invention. Additional examples of such genes are described by accession number in Beigelman *et al.*, USSN 60/363,124, filed March 11, 2002 and incorporated by reference herein in its entirety.

The siNA molecule of the invention can also be used in a variety of agricultural applications involving modulation of endogenous or exogenous gene expression in plants using siNA, including use as insecticidal, antiviral and anti-fungal agents or modulate plant traits such as oil and starch profiles and stress resistance.

Example 16: Diagnostic uses

The siNA molecules of the invention can be used in a variety of diagnostic applications, such as in the identification of molecular targets (e.g., RNA) in a variety of applications, for example, in clinical, industrial, environmental, agricultural and/or research settings. Such diagnostic use of siNA molecules involves utilizing reconstituted RNAi systems, for example, using cellular lysates or partially purified cellular lysates. siNA molecules of this invention can be used as diagnostic tools to examine genetic drift and mutations within diseased cells or to detect the presence of endogenous or exogenous, for example viral, RNA in a cell. The close relationship between siNA activity and the structure of the target RNA allows the detection of mutations in any region of the molecule, which alters the base-pairing and three-dimensional structure of the target RNA. By using multiple siNA molecules described in this invention, one can map nucleotide changes, which are important to RNA structure and function *in vitro*, as well as in cells and tissues. Cleavage of target RNAs with siNA molecules can be used to

inhibit gene expression and define the role of specified gene products in the progression of disease or infection. In this manner, other genetic targets can be defined as important mediators of the disease. These experiments will lead to better treatment of the disease progression by affording the possibility of combination therapies (e.g., multiple siNA molecules targeted to different genes, siNA molecules coupled with known small molecule inhibitors, or intermittent treatment with combinations siNA molecules and/or other chemical or biological molecules). Other *in vitro* uses of siNA molecules of this invention are well known in the art, and include detection of the presence of mRNAs associated with a disease, infection, or related condition. Such RNA is detected by determining the presence of a cleavage product after treatment with a siNA using standard methodologies, for example, fluorescence resonance emission transfer (FRET).

In a specific example, siNA molecules that cleave only wild-type or mutant forms of the target RNA are used for the assay. The first siNA molecules (*i.e.*, those that cleave only wild-type forms of target RNA) are used to identify wild-type RNA present in the sample and the second siNA molecules (*i.e.*, those that cleave only mutant forms of target RNA) are used to identify mutant RNA in the sample. As reaction controls, synthetic substrates of both wild-type and mutant RNA are cleaved by both siNA molecules to demonstrate the relative siNA efficiencies in the reactions and the absence of cleavage of the "non-targeted" RNA species. The cleavage products from the synthetic substrates also serve to generate size markers for the analysis of wild-type and mutant RNAs in the sample population. Thus, each analysis requires two siNA molecules, two substrates and one unknown sample, which is combined into six reactions. The presence of cleavage products is determined using an RNase protection assay so that full-length and cleavage fragments of each RNA can be analyzed in one lane of a polyacrylamide gel. It is not absolutely required to quantify the results to gain insight into the expression of mutant RNAs and putative risk of the desired phenotypic changes in target cells. The expression of mRNA whose protein product is implicated in the development of the phenotype (*i.e.*, disease related or infection related) is adequate to establish risk. If probes of comparable specific activity are used for both transcripts, then a qualitative comparison of RNA levels is adequate and decreases the cost of the initial diagnosis. Higher mutant form to wild-type ratios are correlated with higher risk whether RNA levels are compared qualitatively or quantitatively.

All patents and publications mentioned in the specification are indicative of the levels of skill of those skilled in the art to which the invention pertains. All references cited in this disclosure are incorporated by reference to the same extent as if each reference had been incorporated by reference in its entirety individually.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The methods and compositions described herein as presently representative of preferred embodiments are exemplary and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art, which are encompassed within the spirit of the invention, are defined by the scope of the claims.

It will be readily apparent to one skilled in the art that varying substitutions and modifications can be made to the invention disclosed herein without departing from the scope and spirit of the invention. Thus, such additional embodiments are within the scope of the present invention and the following claims. The present invention teaches one skilled in the art to test various combinations and/or substitutions of chemical modifications described herein toward generating nucleic acid constructs with improved activity for mediating RNAi activity. Such improved activity can comprise improved stability, improved bioavailability, and/or improved activation of cellular responses mediating RNAi. Therefore, the specific embodiments described herein are not limiting and one skilled in the art can readily appreciate that specific combinations of the modifications described herein can be tested without undue experimentation toward identifying siNA molecules with improved RNAi activity.

The invention illustratively described herein suitably can be practiced in the absence of any element or elements, limitation or limitations that are not specifically disclosed herein. Thus, for example, in each instance herein any of the terms "comprising", "consisting essentially of", and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the

scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments, optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and that such modifications and variations are considered to be within the scope of this invention as defined by the description and the appended claims.

In addition, where features or aspects of the invention are described in terms of Markush groups or other grouping of alternatives, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group or other group.

Table I

RPI#	Aliases	Sequence	SEQ ID#
25227	RPI 21550 EGFR 3830L23 AS as siNA Str 1 (sense)	B UAACCUCGUACUGGUGCCUCC B	1
25228	RPI 21550 EGFR 3830L23 AS as siNA Str 2 (antisense)	B GGAGGCACCAGUACGAGGUUA B	2
25229	RPI 21549 EGFR as siNA Str 2 (antisense)	B AAACUCCAAGAUCCCCAAUCA B	3
25230	RPI 21549 EGFR 3 as siNA Str 1 (sense)	B UGAUUGGGGAUCUUGGAGUUU B	4
25231	RPI 21547EGFR as siNA Str 2 (antisense)	B GUUGGAGUCUGUAGGACUUGG B	5
25232	RPI 21547EGFR as siNA Str 1 (sense)	B CCAAGUCCUACAGACUCCAAC B	6
25233	RPI 21545 EGFR as siNA Str 2 (antisense)	B GCAAAAACCCUGUGAUUUCCU B	7
25234	RPI 21545 EGFR as siNA Str 1 (sense)	B AGGAAAUCACAGGGUUUUUGC B	8
25235	RPI 21543 EGFR as siNA Str 2 (antisense)	B UUGGUCAGUUUCUGGCAGUUC B	9
25236	RPI 21543 EGFR as siNA Str 1 (sense)	B GAACUGCCAGAAACUGACCAA B	10
25237	HCV IRES Loop IIIb (Heptazyme site) as siNA str1 (sense)	B GGUCCUUUCUUGGAUCAACCC B	11
25238	HCV IRES Loop IIIb (Heptazyme site) as siNA str2 (antisense)	B GGGUUGAUCCAAGAAAGGACC B	12
25239	HBV (HepBzyme site) as siNA str1(sense)	B UGGACUUCUCUCAAUUUUCUA B	13
25240	HBV (HepBzyme site) as siNA str2 (antisense)	B UAGAAAAUUGAGAGAAGUCCA B	14
25241	HBV18371 site as siNA str1(sense)	B UUUUUCACCUCUGCCUAAUCA B	15
25242	HBV18371 site as siNA str2 (antisense)	B UGAUUAGGCAGAGGUGAAAAA B	16
25243	HBV16372-18373 site as siNA str1(sense)	B CAAGCCUCCAAGCUGUGCCUU B	17
25244	HBV16372-18373 site as siNA str 2 (antisense)	B AAGGCACAGCUUGGAGGCUUG B	18
25245	RPI 17763 Her2Neu AS as siNA Str 2 (antisense)	B UCCAUGGUGCUCACUGCGGCU B	19
25246	RPI 17763 Her2Neu AS as siNA Str 1 (sense)	B AGCCGCAGUGAGCACCAUGGA B	20
25247	RPI 17763 Her2Neu AS as siNA Str 1 (sense) Inverted control	B AGGUACCACGAGUGACGCCGA B	21
25248	RPI 17763 Her2Neu AS as siNA Str 1 (sense) Inverted control compliment	B UCGGCGUCACUCGUGGUACCU B	22
25249	RPI 21550 EGFR 3830L23 AS as siNA Str 1 (sence) Inverted Control	B CCUCCGUGGUCAUGCUCCAAU B	23

25250	RPI 21550 EGFR 3830L23 AS as siNA Str 1 (sence) Inverted Control Compliment	B AUUGGAGCAUGACCACGGAGG B	24
25251	HCV IRES Loop IIIb (Heptazyme site) as siNA str1 (sense) Inverted Control	B CCCAACUAGGUUCUUUCCUGG B	25
25252	HCV IRES Loop IIIb (Heptazyme site) as siNA str1 (sense) Inverted Control Compliment	B CCAGGAAAGAACCUAGUUGGG B	26
25804	RPI 21550 EGFR 3830L23 AS as siNA Str 1 (sense) +2U overhang	UAACCUCGUACUGGUGCCUCCUU	27
25805	RPI 21550 EGFR 3830L23 AS as siNA Str 2 (antisense) +2U overhang	GGAGGCACCAGUACGAGGUUAUU	28
25806	RPI 21549 EGFR as siNA Str 2 (antisense)+ 2U overhang	AAACUCCAAGAUCCCCAAUCAUU	29
25824	RPI 21550 EGFR 3830L23 AS as siNA Str 1 (sense) +2U overhang	BUAACCUCGUACUGGUGCCUCCUUB	30
25825	RPI 21550 EGFR 3830L23 AS as siNA Str 2 (antisense) +2U overhang	BGGAGGCACCAGUACGAGGUUAUUB	31
25826	RPI 21549 EGFR as siNA Str 2 (antisense)+ 2U overhang	BAAACUCCAAGAUCCCCAAUCAUUB	32
25807	RPI 21549 EGFR 3 as siNA Str 1 (sense)+2U overhang	UGAUUGGGGAUCUUGGAGUUUUU	33
25808	RPI 21547EGFR as siNA Str 2 (antisense) +2U overhang	GUUGGAGUCUGUAGGACUUGGUU	34
25809	RPI 21547EGFR as siNA Str 1 (sense) + 2U overhang	CCAAGUCCUACAGACUCCAACUU	35
25827	RPI 21549 EGFR 3 as siNA Str 1 (sense)+2U overhang	BUGAUUGGGGAUCUUGGAGUUUUUB	36
25828	RPI 21547EGFR as siNA Str 2 (antisense) +2U overhang	BGUUGGAGUCUGUAGGACUUGGUUB	37
25829	RPI 21547EGFR as siNA Str 1 (sense) + 2U overhang	BCCAAGUCCUACAGACUCCAACUUB	38
25810	RPI 21545 EGFR as siNA Str 2 (antisense)+2U overhang	GCAAAAACCCUGUGAUUUCCUUU	39
25811	RPI 21545 EGFR as siNA Str 1 (sense)+2U overhang	AGGAAAUCACAGGGUUUUUGCUU	40
25812	RPI 21543 EGFR as siNA Str 2 (antisense)+2U overhang	UUGGUCAGUUUCUGGCAGUUCUU	41

25830	RPI 21545 EGFR as siNA Str 2 (antisense)+2U overhang	BGCAAAAACCCUGUGAUUUCCUUUB	42
25831	RPI 21545 EGFR as siNA Str 1 (sense)+2U overhang	BAGGAAAUCACAGGGUUUUUGCUUB	43
25832	RPI 21543 EGFR as siNA Str 2 (antisense)+2U overhang	BUUGGUCAGUUUCUGGCAGUUCUUB	44
25813	RPI 21543 EGFR as siNA Str 1 (sense)+2U overhang	GAACUGCCAGAAACUGACCAAUU	45
25814	HCV IRES Loop IIIb (Heptazyme site) as siNA str1 (sense)+2U overhang	GGUCCUUUCUUGGAUCAACCCUU	46
25815	HCV IRES Loop IIIb (Heptazyme site) as siNA str2 (antisense) +2U overhang	GGGUUGAUCCAAGAAAGGACCUU	47
25833	RPI 21543 EGFR as siNA Str 1 (sense)+2U overhang	BGAACUGCCAGAAACUGACCAAUUB	48
25834	HCV IRES Loop IIIb (Heptazyme site) as siNA str1 (sense)+2U overhang	BGGUCCUUUCUUGGAUCAACCCUUB	49
25835	HCV IRES Loop IIIb (Heptazyme site) as siNA str2 (antisense) +2U overhang	BGGGUUGAUCCAAGAAAGGACCUUB	50
25816	HBV (HepBzyme site) as siNA str1(sense)+2U overhang	UGGACUUCUCUCAAUUUUCUAUU	51
25817	HBV (HepBzyme site) as siNA str2 (antisense)+2U overhang	UAGAAAAUUGAGAGAAGUCCAUU	52
25818	HBV18371 site as siNA str1(sense)+2U overhang	UUUUUCACCUCUGCCUAAUCAUU	53
25836	HBV (HepBzyme site) as siNA str1(sense)+2U overhang	BUGGACUUCUCUCAAUUUUCUAUUB	54
25837	HBV (HepBzyme site) as siNA str2 (antisense)+2U overhang	BUAGAAAAUUGAGAGAAGUCCAUUB	55
25838	HBV18371 site as siNA str1(sense)+2U overhang	BUUUUUCACCUCUGCCUAAUCAUUB	56
25819	HBV18371 site as siNA str2 (antisense)+2U overhang	UGAUUAGGCAGAGGUGAAAAAUU	57
25820	HBV16372-18373 site as siNA str1(sense)+2U overhang	CAAGCCUCCAAGCUGUGCCUUUU	58
25821	HBV16372-18373 site as siNA str 2 (antisense)+2U overhang	AAGGCACAGCUUGGAGGCUUGUU	59

25839	HBV18371 site as siNA str2 (antisense)+2U overhang	BUGAUUAGGCAGAGGUGAAAAAUUB	60
25840	HBV16372-18373 site as siNA str1(sense)+2U overhang	BCAAGCCUCCAAGCUGUGCCUUUUB	61
25841	HBV16372-18373 site as siNA str 2 (antisense)+2U overhang	BAAGGCACAGCUUGGAGGCUUGUUB	62
25822	RPI 17763 Her2Neu AS as siNA Str 2 (antisense)+2U overhang	UCCAUGGUGCUCACUGCGGCUUU	63
25823	RPI 17763 Her2Neu AS as siNA Str 1 (sense)+2U overhang	AGCCGCAGUGAGCACCAUGGAUU	64
25842	RPI 17763 Her2Neu AS as siNA Str 2 (antisense)+2U overhang	BUCCAUGGUGCUCACUGCGGCUUUB	65
25843	RPI 17763 Her2Neu AS as siNA Str 1 (sense)+2U overhang	BAGCCGCAGUGAGCACCAUGGAUUB	66
27649	RPI GL2 Str1 (sense)	CGUACGCGGAAUACUUCGA TT	67
27650	RPI GL2 Str2 (antisense)	UCGAAGUAUUCCGCGUACG TT	68
27651	RPI Inverted GL2 Str1 (sense)	AGCUUCAUAAGGCGCAUGC TT	69
27652	RPI Inverted GL2 Str2 (antisense)	GCAUGCGCCUUAUGAAGCU TT	70
27653	RPI GL2 Str1 (sense) all ribo P=S	$C_SG_SU_SA_SC_SG_SC_SG_SG_SA_SA_SU_SA_SC_SU_SU_SC_SG_SA$ TT	71
27654	RPI GL2 Str1 (sense) all ribo pyrimidines P=S	$C_SGU_SAC_SGC_SGGAAU_SAC_SU_SU_SC_SGA$ TT	72
27655	RPI GL2 Str1 (sense) 14 5' P=S	$C_SG_SU_SA_SC_SG_SC_SG_SG_SA_SA_SU_SA_SC_S$UUCGA TT	73
27656	RPI GL2 Str1 (sense) 10 5' P=S	$C_SG_SU_SA_SC_SG_SC_SG_SG_SA_S$AUACUUCGA TT	74
27657	RPI GL2 Str1 (sense) 5 5' P=S	$C_SG_SU_SA_SC_S$GCGGAAUACUUCGA TT	75
27658	RPI GL2 Str2 (antisense) all ribo P=S	$U_SC_SG_SA_SA_SG_SU_SA_SU_SU_SC_SC_SG_SC_SG_SU_SA_SC_SG$ TT	76
27659	RPI GL2 Str2 (antisense) all ribo pyrimidines P=S	U_SC_SGAAGU$_S$AU$_S$U$_S$C$_S$C$_S$GC$_S$GU$_S$AC$_S$G TT	77
27660	RPI GL2 Str2 (antisense) 5' 14 P=S	$U_SC_SG_SA_SA_S$GU$_S$A$_S$U$_S$U$_S$C$_S$C$_S$G$_S$C$_S$GUACG TT	78
27661	RPI GL2 Str2 (antisense) 5' 10 P=S	$U_SC_SG_SA_SA_SG_SU_SA_SU_SU_S$CCGCGUACG TT	79
27662	RPI GL2 Str2 (antisense) 5' 5 P=S	$U_SC_SG_SA_SA_S$GUAUUCCGCGUACG TT	80
28010	RPI GL2 Str1 (sense) 5'ligation fragment	CGUACG	81
28011	RPI GL2 Str1 (sense) 3' ligation fragment	CGGAAUACUUCGATT	82

28012	RPI GL2 Str2 (antisense) 5' ligation fragment	UCGAAGUA	83
28013	RPI GL2 Str2 (antisense) 3'ligation fragment	UUCCGCGUACGTT	84
28254	RPI GL2 Str1 (sense) all pyrimidines + TT = PS	$C_sGU_sAC_sGC_sGGAAU_sAC_sU_sU_sC_sGAT_sT$	85
28255	RPI GL2 Str2 (antisense), + TT = PS	$UCGAAGUAUUCCGCGUACGT_sT$	86
28256	RPI GL2 Str2 (antisense), all pyrimidines+ TT = PS	$U_sC_sGAAGU_sAU_sU_sC_sC_sGC_sGU_sAC_sGT_sT$	87
28262	Her2.1.sense Str1 (sense)	UGGGGUCGUCAAAGACGUUTT	88
28263	Her2.1.antisense Str2 (antisense)	AACGUCUUUGACGACCCCATT	89
28264	Her2.1.sense Str1 (sense) inverted	UUGCAGAAACUGCUGGGGUTT	90
28265	Her2.1.antisense Str2 (antisense) inverted	ACCCCAGCAGUUUCUGCAATT	91
28266	Her2.2.sense Str1 (sense)	GGUGCUUGGAUCUGGCGCUTT	92
28267	Her2.2.antisense Str2 (antisense)	AGCGCCAGAUCCAAGCACCTT	93
28268	Her2.2.sense Str1 (sense) inverted	UCGCGGUCUAGGUUCGUGGTT	94
28269	Her2.2.antisense Str2 (antisense) inverted	CCACGAACCUAGACCGCGATT	95
28270	Her2.3.sense Str1 (sense)	GAUCUUUGGGAGCCUGGCATT	96
28271	Her2.3.antisense Str2 (antisense)	UGCCAGGCUCCCAAAGAUCTT	97
28272	Her2.3.sense Str1 (sense) inverted	ACGGUCCGAGGGUUUCUAGTT	98
28273	Her2.3.antisense Str2 (antisense) inverted	CUAGAAACCCUCGGACCGUTT	99
28274	RPI Inverted GL2 Str1 (sense) all ribo pyrimidines P=S	$AGC_sU_sU_sC_sAU_sAAGGC_sGC_sAU_sGC$ TT	100
28275	RPI Inverted GL2 Str1 (sense) 5 5' P=S	$A_sG_sC_sU_sU_sCAUAAGGCGCAUGC$ TT	101
28276	RPI Inverted GL2 Str2 (antisense) all ribo pyrimidines P=S	$GC_sAU_sGC_sGC_sC_sU_sU_sAU_sGAAGC_sU$ TT	102
28277	RPI Inverted GL2 Str2 (antisense) 5 5' P=S	$G_sC_sA_sU_sG_sCGCCUUAUGAAGCU$ TT	103
28278	RPI Inverted GL2 Str2 (antisense) all ribo P=S	$G_sC_sA_sU_sG_sC_sG_sC_sC_sU_sU_sA_sU_sG_sA_sA_sG_sC_sU$ TT	104
28279	RPI Inverted GL2 Str2 (antisense) 14 5' P=S	$G_sC_sA_sU_sG_sC_sG_sC_sC_sU_sU_sA_sU_sG_sAAGCU$ TT	105
28280	RPI Inverted GL2 Str2 (antisense) 10 5' P=S	$G_sC_sA_sU_sG_sC_sG_sC_sC_sU_sUAUGAAGCU$ TT	106

28383	hRelA.1.sense Str1 (sense)	CAGCACAGACCCAGCUGUGTT	107
28384	hRelA.1.antisense Str2 (antisense)	CACAGCUGGGUCUGUGCUGTT	108
28385	hRelA.1.sense Str1 (sense) inverted	GUGUCGACCCAGACACGACTT	109
28386	hRelA.1.antisense Str2 (antisense) inverted	GUCGUGUCUGGGUCGACACTT	110
28387	hRelA.2.sense Str1 (sense)	GCAGGCUGGAGGUAAGGCCTT	111
28388	hRelA.2.antisense Str2 (antisense)	GGCCUUACCUCCAGCCUGCTT	112
28389	hRelA.2.sense Str1 (sense) inverted	CCGGAAUGGAGGUCGGACGTT	113
28390	hRelA.2.antisense Str2 (antisense) inverted	CGUCCGACCUCCAUUCCGGTT	114
28391	h/mRelA.3.sense Str1 (sense)	GACUUCUCCUCCAUUGCGGTT	115
28392	h/mRelA.3.antisense Str2 (antisense)	CCGCAAUGGAGGAGAAGUCTT	116
28393	h/mRelA.3.sense Str1 (sense) inverted	GGCGUUACCUCCUCUUCAGTT	117
28394	h/mRelA.3.antisense Str2 (antisense) inverted	CUGAAGAGGAGGUAACGCCTT	118
28395	h/mRelA.4.sense Str1 (sense)	CACUGCCGAGCUCAAGAUCTT	119
28396	h/mRelA.4.antisense Str2 (antisense)	GAUCUUGAGCUCGGCAGUGTT	120
28397	h/mRelA.4.sense Str1 (sense) inverted	CUAGAACUCGAGCCGUCACTT	121
28398	h/mRelA.4.antisense Str2 (antisense) inverted	GUGACGGCUCGAGUUCUAGTT	122
28399	hIKKg.1.sense Str1 (sense)	GGAGUUCCUCAUGUGCAAGTT	123
28400	hIKKg.1.antisense Str2 (antisense)	CUUGCACAUGAGGAACUCCTT	124
28401	hIKKg.1.sense Str1 (sense) inverted	GAACGUGUACUCCUUGAGGTT	125
28402	hIKKg.1.antisense Str2 (antisense) inverted	CCUCAAGGAGUACACGUUCTT	126
28403	hIKKg.2.sense Str1 (sense)	UCAAGAGCUCCGAGAUGCCTT	127
28404	hIKKg.2.antisense Str2 (antisense)	GGCAUCUCGGAGCUCUUGATT	128
28405	hIKKg.2.sense Str1 (sense) inverted	CCGUAGAGCCUCGAGAACUTT	129
28406	hIKKg.2.antisense Str2 (antisense) inverted	AGUUCUCGAGGCUCUACGGTT	130
28407	h/mIKKG.sense Str1 (sense)	GCAGAUGGCUGAGGACAAGTT	131
28408	h/mIKKG.3.antisense Str2 (antisense)	CUUGUCCUCAGCCAUCUGCTT	132
28409	h/mIKKG.3.sense Str1 (sense) inverted	GAACAGGAGUCGGUAGACGTT	133
28410	h/mIKKG.3.antisense Str2 (antisense) inverted	CGUCUACCGACUCCUGUUCTT	134
28447	RPI construct as hairpin +GAAA+AU blunt	AACGUACGCGGAAUACUUCGAUUAAAAGUAAUCGAAGUAUUCCGCGUACGUU	135

28448	RPI construct as hairpin +GAAA+AU 3' overhang	CGUACGCGGAAUACUUCGAUUAAAAGUAAUCGAAGUAUUCCGCGUACGUU	136
28449	RPI construct as hairpin +GAAA blunt	AACGUACGCGGAAUACUUCGAUUAAAGAAUCGAAGUAUUCCGCGUACGUU	137
28450	RPI construct as hairpin +GAAA 3' overhang	CGUACGCGGAAUACUUCGAUUAAAGAAUCGAAGUAUUCCGCGUACGUU	138
28451	RPI construct as hairpin +UUG 3' overhang	CGUACGCGGAAUACUUCGAUUGUUAAUCGAAGUAUUCCGCGUACGUU	139
28452	RPI construct as hairpin +UUG blunt	AACGUACGCGGAAUACUUCGAUUGUUAAUCGAAGUAUUCCGCGUACGUU	140
28453	RPI construct as hairpin +UUG+AU blunt	AACGUACGCGGAAUACUUCGAUUAGUUUAAUCGAAGUAUUCCGCGUACGUU	141
28454	RPI construct as hairpin +UUG 3' overhang	CGUACGCGGAAUACUUCGAUUAGUUUAAUCGAAGUAUUCCGCGUACGUU	142
28415	HCV-Luc:325U21 TT siNA (sense)	CCCCGGGAGGUCUCGUAGATT	143
28416	HCV-Luc:162U21 TT siNA (sense)	CGGAACCGGUGAGUACACCTT	144
28417	HCV-Luc:324U21 TT siNA (sense)	GCCCCGGGAGGUCUCGUAGTT	145
28418	HCV-Luc:163U21 TT siNA (sense)	GGAACCGGUGAGUACACCGTT	146
28419	HCV-Luc:294U21 TT siNA (sense)	GUGGUACUGCCUGAUAGGGTT	147
28420	HCV-Luc:293U21 TT siNA (sense)	UGUGGUACUGCCUGAUAGGTT	148
28421	HCV-Luc:292U21 TT siNA (sense)	UUGUGGUACUGCCUGAUAGTT	149
28422	HCV-Luc:343L21 TT siNA (325C) (antisense)	UCUACGAGACCUCCCGGGGTT	150
28423	HCV-Luc:180L21 TT siNA (162C) (antisense)	GGUGUACUCACCGGUUCCGTT	151
28424	HCV-Luc:342L21 TT siNA (324C) (antisense)	CUACGAGACCUCCCGGGGCTT	152
28425	HCV-Luc:181L21 TT siNA (163C) (antisense)	CGGUGUACUCACCGGUUCCTT	153
28426	HCV-Luc:312L21 TT siNA (294C) (antisense)	CCCUAUCAGGCAGUACCACTT	154
28427	HCV-Luc:311L21 TT siNA (293C) (antisense)	CCUAUCAGGCAGUACCACATT	155
28428	HCV-Luc:310L21 TT siNA (292C) (antisense)	CUAUCAGGCAGUACCACAATT	156
28429	HCV-Luc:325U21 TT siNA (sense) inv	TTAGAUGCUCUGGAGGGCCCC	157
28430	HCV-Luc:162U21 TT siNA (sense) inv	TTCCACAUGAGUGGCCAAGGC	158
28431	HCV-Luc:324U21 TT siNA (sense) inv	TTGAUGCUCUGGAGGGCCCCG	159
28432	HCV-Luc:163U21 TT siNA (sense) inv	TTGCCACAUGAGUGGCCAAGG	160
28433	HCV-Luc:294U21 TT siNA (sense) inv	TTGGGAUAGUCCGUCAUGGUG	161
28434	HCV-Luc:293U21 TT siNA (sense) inv	TTGGAUAGUCCGUCAUGGUGU	162
28435	HCV-Luc:292U21 TT siNA (sense) inv	TTGAUAGUCCGUCAUGGUGUU	163
28436	HCV-Luc:343L21 TT siNA (325C) (antisense) inv	TTGGGGCCCUCCAGAGCAUCU	164
28437	HCV-Luc:180L21 TT siNA (162C)	TTGCCUUGGCCACUCAUGUGG	165

	(antisense) inv		
28438	HCV-Luc:342L21 TT siNA (324C) (antisense) inv	TTCGGGGCCCUCCAGAGCAUC	166
28439	HCV-Luc:181L21 TT siNA (163C) (antisense) inv	TTCCUUGGCCACUCAUGUGGC	167
28440	HCV-Luc:312L21 TT siNA (294C) (antisense) inv	TTCACCAUGACGGACUAUCCC	168
28441	HCV-Luc:311L21 TT siNA (293C) (antisense) inv	TTACACCAUGACGGACUAUCC	169
28442	HCV-Luc:310L21 TT siNA (292C) (antisense) inv	TTAACACCAUGACGGACUAUC	170
28458	RPI Inverted GL2 Str1 (sense) 5 5' P=S + TsT	$A_SG_SC_SU_SU_S$CAUAAGGCGCAUGC T_ST	171
28459	RPI Inverted GL2 Str2 (antisense) 5 5' P=S + TsT	$G_SC_SA_SU_SG_S$CGCCUUAUGAAGCU T_ST	172
28460	RPI GL2 Str1 (sense) 5 5' P=S + TsT	$C_SG_SU_SA_SC_S$GCGGAAUACUUCGA T_ST	173
28461	RPI GL2 Str2 (antisense) 5 5' P=S + TsT	$U_SC_SG_SA_SA_S$GUAUUCCGCGUACG T_ST	174
28511	RPI GL2 Str2 (antisense) + RPI GL2 Str1 (sense) (tandem synth. w/ idB on 3' of Str 1)	CGUACGCGGAAUACUUCGATTBUCGAAGUAUUCCGCGUACG TT	175
29543	HBV:248U21 siNA pos (sense)	GUCUAGACUCGUGGUGGACTT	176
29544	HBV:414U21 siNA pos (sense)	CCUGCUGCUAUGCCUCAUCTT	177
29545	HBV:1867U21 siNA pos (sense)	CAAGCCUCCAAGCUGUGCCTT	178
29546	HBV:1877U21 siNA pos (sense)	AGCUGUGCCUUGGGUGGCUTT	179
29547	HBV:228L21 siNA neg (248C) (antisense)	GUCCACCACGAGUCUAGACTT	180
29548	HBV:394L21 siNA neg (414C) (antisense)	GAUGAGGCAUAGCAGCAGGTT	181
29549	HBV:1847L21 siNA neg (1867C) (antisense)	GGCACAGCUUGGAGGCUUGTT	182
29550	HBV:1857L21 siNA neg (1877C) (antisense)	AGCCACCCAAGGCACAGCUTT	183
29551	HBV:248U21 siNA pos (sense) inv	CAGGUGGUGCUCAGAUCUGTT	184
29552	HBV:414U21 siNA pos (sense) inv	CUACUCCGUAUCGUCGUCCTT	185
29553	HBV:1867U21 siNA pos (sense) inv	CCGUGUCGAACCUCCGAACTT	186
29554	HBV:1877U21 siNA pos (sense) inv	UCGGUGGGUUCCGUGUCGATT	187
29555	HBV:228L21 siNA neg (248C) (antisense) inv	CAGAUCUGAGCACCACCUGTT	188
29556	HBV:394L21 siNA neg (414C) (antisense) inv	GGACGACGAUACGGAGUAGTT	189
29557	HBV:1847L21 siNA neg (1867C) (antisense) inv	GUUCGGAGGUUCGACACGGTT	190
29558	HBV:1857L21 siNA neg (1877C) (antisense) inv	UCGACACGGAACCCACCGATT	191

29573	HCV-Luc:162U21 siNA (sense)	CGGAACCGGUGAGUACACCGG	192
29574	HCV-Luc:163U21 siNA (sense)	GGAACCGGUGAGUACACCGGA	193
29575	HCV-Luc:292U21 siNA (sense)	UUGUGGUACUGCCUGAUAGGG	194
29576	HCV-Luc:293U21 siNA (sense)	UGUGGUACUGCCUGAUAGGGU	195
29577	HCV-Luc:294U21 siNA (sense)	GUGGUACUGCCUGAUAGGGUG	196
29578	HCV-Luc:324U21 siNA (sense)	GCCCCGGGAGGUCUCGUAGAC	197
29579	HCV-Luc:325U21 siNA (sense)	CCCCGGGAGGUCUCGUAGACC	198
29580	HCV-Luc:182L21 siNA (162C) (antisense)	GGUGUACUCACCGGUUCCGCA	199
29581	HCV-Luc:183L21 siNA (163C) (antisense)	CGGUGUACUCACCGGUUCCGC	200
29582	HCV-Luc:312L21 siNA (292C) (antisense)	CUAUCAGGCAGUACCACAAGG	201
29583	HCV-Luc:313L21 siNA (293C) (antisense)	CCUAUCAGGCAGUACCACAAG	202
29584	HCV-Luc:314L21 siNA (294C) (antisense)	CCCUAUCAGGCAGUACCACAA	203
29585	HCV-Luc:344L21 siNA (324C) (antisense)	CUACGAGACCUCCCGGGGCAC	204
29586	HCV-Luc:345L21 siNA (325C) (antisense)	UCUACGAGACCUCCCGGGGCA	205
29587	HCV-Luc:162U21 siNA (sense) rev	GGCCACAUGAGUGGCCAAGGC	206
29588	HCV-Luc:163U21 siNA (sense) rev	AGGCCACAUGAGUGGCCAAGG	207
29589	HCV-Luc:292U21 siNA (sense) rev	GGGAUAGUCCGUCAUGGUGUU	208
29590	HCV-Luc:293U21 siNA (sense) rev	UGGGAUAGUCCGUCAUGGUGU	209
29591	HCV-Luc:294U21 siNA (sense) rev	GUGGGAUAGUCCGUCAUGGUG	210
29592	HCV-Luc:324U21 siNA (sense) rev	CAGAUGCUCUGGAGGGCCCCG	211
29593	HCV-Luc:325U21 siNA (sense) rev	CCAGAUGCUCUGGAGGGCCCC	212
29594	HCV-Luc:182L21 siNA (162C) (antisense) rev	ACGCCUUGGCCACUCAUGUGG	213
29595	HCV-Luc:183L21 siNA (163C) (antisense) rev	CGCCUUGGCCACUCAUGUGGC	214
29596	HCV-Luc:312L21 siNA (292C) (antisense) rev	GGAACACCAUGACGGACUAUC	215

29597	HCV-Luc:313L21 siNA (293C) (antisense) rev	GAACACCAUGACGGACUAUCC	216
29598	HCV-Luc:314L21 siNA (294C) (antisense) rev	AACACCAUGACGGACUAUCCC	217
29599	HCV-Luc:344L21 siNA (324C) (antisense) rev	CACGGGGCCCUCCAGAGCAUC	218
29600	HCV-Luc:345L21 siNA (325C) (antisense) rev	ACGGGGCCCUCCAGAGCAUCU	219
29601	Luc2:128U21 siNA (sense)	CAGAUGCACAUAUCGAGGUGA	220
29602	Luc3:128U21 siNA (sense)	CAGAUGCACAUAUCGAGGUGG	221
29603	Luc2/3:128U21 TT siNA (sense)	CAGAUGCACAUAUCGAGGUTT	222
29604	Luc2/3:148L21 siNA (128C) (antisense)	ACCUCGAUAUGUGCAUCUGUA	223
29605	Luc2/3:148L21 TT siNA (128C) (antisense)	ACCUCGAUAUGUGCAUCUGTT	224
29606	Luc2/3:166U21 siNA (sense)	UACUUCGAAAUGUCCGUUCGG	225
29607	Luc2/3:166U21 TT siNA (sense)	UACUUCGAAAUGUCCGUUCTT	226
29608	Luc2:186L21 siNA (166C) (antisense)	GAACGGACAUUUCGAAGUAUU	227
29609	Luc3:186L21 siNA (166C) (antisense)	GAACGGACAUUUCGAAGUACU	228
29610	Luc2/3:186L21 TT siNA (166C) (antisense)	GAACGGACAUUUCGAAGUATT	229
29611	Luc2/3:167U21 siNA (sense)	ACUUCGAAAUGUCCGUUCGGU	230
29612	Luc2/3:167U21 TT siNA (sense)	ACUUCGAAAUGUCCGUUCGTT	231
29613	Luc2:187L21 siNA (167C) (antisense)	CGAACGGACAUUUCGAAGUAU	232
29614	Luc3:187L21 siNA (167C) (antisense)	CGAACGGACAUUUCGAAGUAC	233
29615	Luc2/3:187L21 TT siNA (167C) (antisense)	CGAACGGACAUUUCGAAGUTT	234
29616	Luc2/3:652U21 siNA (sense)	AGAUUCUCGCAUGCCAGAGAU	235
29617	Luc2/3:652U21 TT siNA (sense)	AGAUUCUCGCAUGCCAGAGTT	236
29618	Luc2:672L21 siNA (652C) (antisense)	CUCUGGCAUGCGAGAAUCUGA	237
29619	Luc3:672L21 siNA (652C) (antisense)	CUCUGGCAUGCGAGAAUCUCA	238
29620	Luc2/3:672L21 TT siNA (652C) (antisense)	CUCUGGCAUGCGAGAAUCUTT	239
29621	Luc2/3:653U21 siNA (sense)	GAUUCUCGCAUGCCAGAGAUC	240
29622	Luc2/3:653U21 TT siNA (sense)	GAUUCUCGCAUGCCAGAGATT	241
29623	Luc2:673L21 siNA (653C) (antisense)	UCUCUGGCAUGCGAGAAUCUG	242
29624	Luc3:673L21 siNA (653C) (antisense)	UCUCUGGCAUGCGAGAAUCUC	243
29625	Luc2/3:673L21 TT siNA (653C) (antisense)	UCUCUGGCAUGCGAGAAUCTT	244

29626	Luc2/3:880U21 siNA (sense)	UUCUUCGCCAAAAGCACUCUG	245
29627	Luc2/3:880U21 TT siNA (sense)	UUCUUCGCCAAAAGCACUCTT	246
29628	Luc2:900L21 siNA (880C) (antisense)	GAGUGCUUUUGGCGAAGAAUG	247
29629	Luc3:900L21 siNA (880C) (antisense)	GAGUGCUUUUGGCGAAGAAGG	248
29630	Luc2/3:900L21 TT siNA (880C) (antisense)	GAGUGCUUUUGGCGAAGAATT	249
29631	Luc2/3:1012U21 siNA (sense)	CAAGGAUAUGGGCUCACUGAG	250
29632	Luc2/3:1012U21 TT siNA (sense)	CAAGGAUAUGGGCUCACUGTT	251
29633	Luc2:1032L21 siNA (1012C) (antisense)	CAGUGAGCCCAUAUCCUUGUC	252
29634	Luc3:1032L21 siNA (1012C) (antisense)	CAGUGAGCCCAUAUCCUUGCC	253
29635	Luc2/3:1032L21 TT siNA (1012C) (antisense)	CAGUGAGCCCAUAUCCUUGTT	254
29636	Luc2:1139U21 siNA (sense)	AAACGCUGGGCGUUAAUCAGA	255
29637	Luc3:1139U21 siNA (sense)	AAACGCUGGGCGUUAAUCAAA	256
29638	Luc2/3:1139U21 TT siNA (sense)	AAACGCUGGGCGUUAAUCATT	257
29639	Luc2/3:1159L21 siNA (1139C) (antisense)	UGAUUAACGCCCAGCGUUUUC	258
29640	Luc2/3:1159L21 TT siNA (1139C) (antisense)	UGAUUAACGCCCAGCGUUUTT	259
29641	Luc2:1283U21 siNA (sense)	AAGACGAACACUUCUUCAUAG	260
29642	Luc3:1283U21 siNA (sense)	AAGACGAACACUUCUUCAUCG	261
29643	Luc2/3:1283U21 TT siNA (sense)	AAGACGAACACUUCUUCAUTT	262
29644	Luc2/3:1303L21 siNA (1283C) (antisense)	AUGAAGAAGUGUUCGUCUUCG	263
29645	Luc2/3:1303L21 TT siNA (1283C) (antisense)	AUGAAGAAGUGUUCGUCUUTT	264
29646	Luc2:1487U21 siNA (sense)	AAGAGAUCGUGGAUUACGUGG	265
29647	Luc3:1487U21 siNA (sense)	AAGAGAUCGUGGAUUACGUCG	266
29648	Luc2/3:1487U21 TT siNA (sense)	AAGAGAUCGUGGAUUACGUTT	267
29649	Luc2/3:1507L21 siNA (1487C) (antisense)	ACGUAAUCCACGAUCUCUUUU	268
29650	Luc2/3:1507L21 TT siNA (1487C) (antisense)	ACGUAAUCCACGAUCUCUUTT	269
29651	Luc2:1622U21 siNA (sense)	AGGCCAAGAAGGGCGGAAAGU	270
29652	Luc3:1622U21 siNA (sense)	AGGCCAAGAAGGGCGGAAAGA	271
29653	Luc2/3:1622U21 TT siNA (sense)	AGGCCAAGAAGGGCGGAAATT	272

29654	Luc2/3:1642L21 siNA (1622C) (antisense)	UUUCCGCCCUUCUUGGCCUUU	273
29655	Luc2/3:1642L21 TT siNA (1622C) (antisense)	UUUCCGCCCUUCUUGGCCUTT	274
29656	Luc2:1623U21 siNA (sense)	GGCCAAGAAGGGCGGAAAGUC	275
29657	Luc3:1623U21 siNA (sense)	GGCCAAGAAGGGCGGAAAGAU	276
29658	Luc2/3:1623U21 TT siNA (sense)	GGCCAAGAAGGGCGGAAAGTT	277
29659	Luc2/3:1643L21 siNA (1623C) (antisense)	CUUUCCGCCCUUCUUGGCCUU	278
29660	Luc2/3:1643L21 TT siNA (1623C) (antisense)	CUUUCCGCCCUUCUUGGCCTT	279
29663	RPI GL2 Str2 (antisense), all pyrimidines+ 5BrdUT = PS	$U_SC_SGAAGU_SAU_SU_SC_SC_SGC_SGU_SAC_SGU_ST$	280
29664	RPI GL2 Str1 (sense) all pyrimidines + 5-BrdUT = PS	$C_SGU_SAC_SGC_SGGAAU_SAC_SU_SU_SC_SGAU_ST$	281
29665	RPI GL2 Str1 (sense) 5 5' +5-BrdUT = P=S	$C_SG_SU_SA_SC_S$GCGGAAUACUUCGA U_ST	282
29666	RPI GL2 Str2 (antisense) 5' 5 +5BrdUT= P=S	$U_SC_SG_SA_SA_S$GUAUUCCGCGUACG U_ST	283
29667	RPI GL2 Str1 (sense) all pyrimidines + TT = PS+3'invAba	$C_SGU_SAC_SGC_SGGAAU_SAC_SU_SU_SC_SGAT_STB$	284
29668	RPI GL2 Str1 (sense) all pyrimidines = PS+3' and 5' nvAba	$BC_SGU_SAC_SGC_SGGAAU_SAC_SU_SU_SC_SGAT_STB$	285
29669	RPI GL2 Str1 (sense) all pyrimidines + TT = PS+ 5' invAba	$BC_SGU_SAC_SGC_SGGAAU_SAC_SU_SU_SC_SGAT_ST$	286
29670	RPI GL2 Str2 (antisense), all pyrimidines +TT = PS + 3'inverted *abasic*	$U_SC_SGAAGU_SAU_SU_SC_SC_SGC_SGU_SAC_SGT_STB$	287
29671	RPI GL2 Str2 (antisense), all pyrimidines +TT = PS + 3'and 5' inverted *abasic*	$BU_SC_SGAAGU_SAU_SU_SC_SC_SGC_SGU_SAC_SGT_STB$	288
29672	RPI GL2 Str2 (antisense), all pyrimidines +TT = PS + 5' inverted *abasic*	$BU_SC_SGAAGU_SAU_SU_SC_SC_SGC_SGU_SAC_SGT_ST$	289
29678	RPI GL2 Str1 (sense) + RPI GL2 Str2 (antisense) (tandem synth. w/ idB on 3' of Str 2)	UCGAAGUAUUCCGCGUACG TTBCGUACGCGGAAUACUUCGATT	290
29681	RPI GL2 Str1 (sense) 5'ligation fragment 5-5'-P=S	$C_SG_SU_SA_SC_SG$	291
29682	RPI GL2 Str1 (sense) 3'-ligation fragment 5-5'-P=S	CGGAAUACUUCGAT$_S$T	292
29683	RPI GL2 Str2 (antisense) 5' ligation fragment 5-5'-P=S	$U_SC_SG_SA_SA_S$GUA	293
29684	RPI GL2 Str2 (antisense) 3' ligation fragment 5-5'-P=S	UUCCGCGUACGT$_S$T	294
29685	RPI GL2 Str2 (antisense) 5' ligation fragment all-P=S	$U_SC_SG_SA_SA_SG_SU_SA$	295
29686	RPI GL2 Str2 (antisense) 3' ligation	$U_SU_SC_SC_SG_SC_SG_SU_SA_SC_SG_ST_ST$	296

	fragment all-P=S		
29694	FLT1:349U21 siNA stab1 (sense)	$C_sU_sG_sA_sG_sUUUAAAAGGCACCCT_sT$	297
29695	FLT1:2340U21 siNA stab1 (sense)	$C_sA_sA_sC_sC_sACAAAAUACAACAAT_sT$	298
29696	FLT1:3912U21 siNA stab1 (sense)	$C_sC_sU_sG_sG_sAAAGAAUCAAAACCT_sT$	299
29697	FLT1:2949U21 siNA stab1 (sense)	$G_sC_sA_sA_sG_sGAGGGCCUCUGAUGT_sT$	300
29698	FLT1:369L21 siNA (349C) stab1 (sense)	$G_sG_sG_sU_sG_sCCUUUUAAACUCAGT_sT$	301
29699	FLT1:2360L21 siNA (2340C) stab1 (sense)	$U_sU_sG_sU_sU_sGUAUUUUGUGGUUGT_sT$	302
29700	FLT1:3932L21 siNA (3912C) stab1 (sense)	$G_sG_sU_sU_sU_sUGAUUCUUUCCAGGT_sT$	303
29701	FLT1:2969L21 siNA (2949C) stab1 (sense)	$C_sA_sU_sC_sA_sGAGGCCCUCCUUGCT_sT$	304
29706	FLT1:369L21 siNA (349C) (antisense) stab2	$G_sG_sG_sU_sG_sC_sC_sU_sU_sU_sU_sA_sA_sA_sC_sU_sC_sA_sG_sT_sT$	305
29707	FLT1:2360L21 siNA (2340C) (antisense) stab2	$U_sU_sG_sU_sU_sG_sU_sA_sU_sU_sU_sU_sG_sU_sG_sG_sU_sU_sG_sT_sT$	306
29708	FLT1:3932L21 siNA (3912C) (antisense) stab2	$G_sG_sU_sU_sU_sU_sG_sA_sU_sU_sC_sU_sU_sU_sC_sC_sA_sG_sG_sT_sT$	307
29709	FLT1:2969L21 siNA (2949C) (antisense) stab2	$C_sA_sU_sC_sA_sG_sA_sG_sG_sC_sC_sC_sU_sC_sC_sU_sU_sG_sC_sT_sT$	308
28030	RPI GL2 Str1 (sense)	ggcauuggccaacguacgcggaauacuucgauucgguuacgaa	309
28242	RPI GL2 Str1 (sense) 2'-OMe	cguacgcggaauacuucgauu	310
28243	RPI GL2 Str1 (sense) 14 5' 2'-O-Me	cguacgcggaauacUUCGATT	311
28244	RPI GL2 Str1 (sense) 10 5' 2'-O-Me	cguacgcggaAUACUUCGATT	312
28245	RPI GL2 Str1 (sense) 5 5' 2'-O-Me	cguacGCGGAAUACUUCGATT	313
28246	RPI GL2 Str2 (antisense) all 2'-O-me	ucgaaguauuccgcguacguu	314
28247	RPI GL2 Str2 (antisense) all ribo pyrimidines = 2'-Ome	ucGAAGuAuuccGcGuAcGuu	315
28248	RPI GL2 Str2 (antisense) 5' 14 2'-O-Me	ucgaaguauuccgcGUACGTT	316
28249	RPI GL2 Str2 (antisense) 5' 10 2'-O-Me	ucgaaguauuCCGCGUACGTT	317
28250	RPI GL2 Str2 (antisense) 5' 2'-O-Me	ucgaaGUAUUCCGCGUACGTT	318
28251	RPI GL2 Str1 (sense) all pyrimidines 2'-O-Me except 3'-TT	cGuAcGcGGAAuAcuucGATT	319

28252	RPI GL2 Str1 (sense) all pyrimidines = 2'-OMe	cGuAcGcGGAAuAcuucGAuu	320
28253	RPI GL2 Str1 (sense)+ TT =P=S	CGUACGCGGAAUACUUCGAT$_S$T	321
28261	RPI GL2 Str2 (antisense) all ribo pyrimidines = 2'-O-me,except 3'-TT	ucGAAGuAuuccGcGuAcGTT	322
28257	RPI GL2 Str1 (sense)+ 3' univ. base 2	CGUACGCGGAAUACUUCGAXX	323
28258	RPI GL2 Str1 (sense)+ 3' univ base 1	CGUACGCGGAAUACUUCGAZZ	324
28259	RPI GL2 Str2 (antisense), + 3' univ. base 2	UCGAAGUAUUCCGCGUACGXX	325
28260	RPI GL2 Str2 (antisense), + 3' univ. base 1	UCGAAGUAUUCCGCGUACGZZ	326
28014	RPI GL2 Str1 (sense) 5'ligation fragment P=Scapped Y-2'F	c_SG$_S$$u_SA_S$$c$G	327
28015	RPI GL2 Str1 (sense) 3' ligation fragment P=Scapped Y-2'F	cGGAAuAcuuc_SG$_S$A$_S$T$_S$T	328
28026	RPI GL2 Str1 (sense)P=Scapped Y-2'F	c_SG$_S$$u_SA_S$$cGc$GGAA$uAcuuc_SG_SA_ST_S$T	329
28016	RPI GL2 Str2 (antisense) 5' ligation fragment P=Scapped Y-2'F	$u_S$$c_SG_SA_SAGu$A	330
28017	RPI GL2 Str2 (antisense) 3'ligation fragment P=Scapped Y-2'F	$uucc$GCGuA$_S$$c_SG_ST_S$T	331
28027	RPI GL2 Str2 (antisense) P=Scapped Y-2'F	$u_S$$c_SG_SA_SAGuAuuccGCGuA_S$$c_SG_ST_S$T	332
28018	RPI GL2 Str1 (sense) 5'ligation fragment 5'P=S Y-2'F	$_S$$cGuAcG$	333
28019	RPI GL2 Str1 (sense) 3' ligation fragment 5'P=S Y-2'F	cGGAAuAcuucGATT	334
28028	RPI GL2 Str1 (sense)5'P=S Y-2'F	$_S$$cGuAcGcGGAAuAcuuc$GATT	335
28020	RPI GL2 Str2 (antisense) 5' ligation fragment 5'P=S Y-2'F	$_S$$uc$GAAG$u$A	336
28021	RPI GL2 Str2 (antisense) 3'ligation fragment 5'P=S Y-2'F	$uucc$GCGuAcGTT	337
28029	RPI GL2 Str2 (antisense) 5'P=S Y-2'F	$_S$$uc$GAAG$uAuuccGCGu$AcGTT	338
28022	RPI Inverted GL2 Str1 (sense) P=Scapped Y-2'F	A$_S$G$_S$$c_S$$u_S$$uc$A$u$AAGGc$GcAu_SG_S$$c_ST_S$T	339
28023	RPI Inverted GL2 Str2 (antisense) P=Scapped Y-2'F	G$_S$$c_SA_S$$u_SGcGccuu$A$u$GAAG$_S$$c_S$$u_ST_S$T	340
28024	RPI Inverted GL2 Str1 (sense) 5'P=S Y-2'F	$_S$AG$cuuc$AuAAGGcGcAuGcTT	341

28025	RPI Inverted GL2 Str2 (antisense) 5'P=S Y-2'F	$_{S}$GcAuGcGccuuAuGAAGcuTT	342
28455	RPI GL2 Str1 (sense) 2'-F U C	cGuAcGcGGAAuAcuucGATT	343
28456	RPI GL2 Str2 (antisense) 2'-F U C	ucGAAGuAuuccGcGuAcGTT	344
29702	FLT1:349U21 siNA stab3 (sense)	$c_su_sG_sA_s$GuuuAAAAGGcAc$_sc_sc_sT_sT$	345
29703	FLT1:2340U21 siNA stab3 (sense)	$c_sA_sA_sc_s$cAcAAAAuAcAAc$_sA_sA_sT_sT$	346
29704	FLT1:3912U21 siNA stab3 (sense)	$c_sc_su_sG_s$GAAAGAAucAAAA$_sc_sc_sT_sT$	347
29705	FLT1:2949U21 siNA stab3 (sense)	$G_sc_sA_sA_s$GGAGGGccucuGA$_su_sG_sT_sT$	348
28443	RPI GL2 Str1 (sense) 2'-amino U C	cGuAcGcGGAAuAcuucGATT	349
28444	RPI GL2 Str2 (antisense) 2'-amino U C	ucGAAGuAuuccGcGuAcGTT	350
28445	RPI GL2 Str1 (sense) 2'-amino U C uT 3'end	cGuAcGcGGAAuAcuucGAuT	351
28446	RPI GL2 Str2 (antisense) 2'-amino U C uT 3'end	ucGAAGuAuuccGcGuAcGuT	352
30051	HCV-Luc:325U21 siNA 5 5' P=S + 3' univ. base 2 + 5'/3' invAba (antisense)	BC$_sC_sC_sC_sG_s$GGAGGUCUCGUAGAXXB	353
30052	HCV-Luc:325U21 siNA rev 5 5' P=S + 3' univ. base 2 + 5'/3' invAba (antisense)	BA$_sG_sA_sU_sG_s$CUCUGGAGGGCCCCXXB	354
30053	HCV-Luc:345L21 siNA (325C) (antisense) 5 5' P=S + 3' univ. base 2 + 3' invAba (sense)	U$_sC_sU_sA_sC_s$GAGACCUCCCGGGGXXB	355
30054	HCV-Luc:345L21 siNA (325C) (antisense) rev 5 5' P=S + 3' univ. base 2 + 3' invAba (sense)	G$_sG_sG_sG_sC_s$CCUCCAGAGCAUCUXXB	356
30055	HCV-Luc:325U21 siNA all Y P=S + 3' univ. base 2 + 5'/3' invAba (antisense)	BC$_sC_sC_sC_s$GGGAGGU$_sC_sU_sC_s$GU$_s$AGAXXB	357
30056	HCV-Luc:325U21 siNA rev all Y P=S + 3' univ. base 2 + 5'/3' invAba (antisense)	BAGAU$_s$GC$_sU_sC_sU_s$GGAGGGC$_sC_sC_sC_s$XXB	358
30057	HCV-Luc:345L21 siNA (325C) (antisense) all Y P=S + 3' univ. base 2 + 3' invAba (sense)	U$_sC_sU_s$AC$_s$GAGAC$_sC_sU_sC_sC_sC_s$GGGGXXB	359
30058	HCV-Luc:345L21 siNA (325C) (antisense) rev all Y P=S + 3' univ. base 2 + 3' invAba (sense)	GGGGC$_sC_sC_sU_sC_sC_s$AGAGC$_s$AU$_sC_sU_s$XXB	360

30059	HCV-Luc:325U21 siNA 4/3 P=S ends + all Y-2'F + 3' univ. base 2 + 5'/3' invAba (antisense)	$Bc_sc_sc_sc_sGGGAGGucucGuA_sG_sA_sXXB$	361
30060	HCV-Luc:325U21 siNA rev 4/3 P=S ends + all Y-2'F + 3' univ. base 2 + 5'/3' invAba (antisense)	$BA_sG_sA_su_sGcucuGGAGGGcc_sc_sc_sXXB$	362
30170	HCV-Luc:325U21 siNA all Y-2'F + 3' univ. base 2 + 5'/3' invAba (antisense)	B *cccc*GGGAGG*ucuc*Gu*A*GAXX B	363
30171	HCV-Luc:325U21 siNA rev all Y-2'F + 3' univ. base 2 + 5'/3' invAba (antisense)	B AGA*uGcucu*GGAGGG*cccc*XX B	364
30172	HCV-Luc:345L21 siNA (325C) (antisense) all Y P=S + 3' univ. base 2 + 5'/3' invAba (antisense)	B $U_sC_sU_sAC_sGAGAC_sC_sU_sC_sC_sC_sGGGGXX$ B	365
30173	HCV-Luc:345L21 siNA (325C) (antisense) all Y-2'F	*ucu*AcGAGA*ccucccc*GGGG	366
30174	HCV-Luc:345L21 siNA (325C) (antisense) rev all Y-2'F	GGGG*cccucc*AGAGc*Aucu*	367
30175	HCV-Luc:345L21 siNA (325C) (antisense) all Y-2'F + 3' univ. base 2	*ucu*AcGAGA*ccucccc*GGGGXX	368
30176	HCV-Luc:345L21 siNA (325C) (antisense) rev all Y-2'F + 3' univ. base 2	GGGG*cccucc*AGAGc*Aucu*XX	369
30177	HCV-Luc:345L21 siNA (325C) (antisense) all Y-2'F + 3' univ. base 2 + 5'/3' iB	B *ucu*AcGAGA*ccucccc*GGGGXX B	370
30178	HCV-Luc:325U21 siNA all Y P=S + 3' univ. base 2 + 3' invAba (sense)	$C_sC_sC_sC_sGGGAGGU_sC_sU_sC_sGU_sAGAXX$ B	371
30063	RPI GL2 Str1 (sense) 2'-F U,C + 3' ,5' *abasic*	Bc*Gu*Ac*Gc*GGAA*u*Acu*uc*GATTB	372
30222	RPI GL2 Str1 (sense) Y 2'-O-Me with 3'-TT & 5'/3' iB	B cGuAcGcGGAAuAcuucGATT B	373
30224	RPI GL2 Str2 (antisense) Y 2'-F & 3' TsT	*uc*GAAG*u*A*uuccGcGu*AcGT$_s$T	374
30430	RPI GL2 Str2 (antisense) 2'-F U,C + 5',3' abasic, A,G= 2'-O-Me	*uc*gaag*uauuccgcgu*acgT$_s$T	375
30431	RPI GL2 Str1 (sense) 2'-F U,C + 3' ,5' abasic,TT; 2'-O-Me-A,G	Bc*guacgcggaauacuuc*gaTTB	376
30433	RPI GL2 Str1 (sense) 2'-F U,C + 3' ,5' abasic,TT; 2'-deoxy-A,G	Bc*GuAcGcGGAAuAcuucGA*TTB	377
30550	RPI GL2 Str2 (antisense) 2'-F U,C 3'-dTsT	*uc*GAAG*u*A*uuccGcGu*AcGT$_s$t	378

30555	RPI GL2 Str2 (antisense) 2'-F U,C 3'-glycerol.T	*uc*GAAG*u*A*uuccGcGu*AcGTL	379
30556	RPI GL2 Str2 (antisense) 2'-F U,C 3'-glycerol,2T	*uc*GAAG*u*A*uuccGcGu*AcGTTL	380
30226	rev RPI GL2 Str1 (sense) Y 2'-O-Me with 3'-TT & 5'/3' iB	B AGcuucAuAAGGcGcAuGcTT B	381
30227	rev RPI GL2 Str1 (sense) Y 2'-F with 3'-TT & 5'/3' iB	B AG*cuucAu*AAGGcGc*Au*GcTT B	382
30229	rev RPI GL2 Str2 (antisense) Y 2'-F & 3' TsT	GcA*u*GcGc*cuu*A*u*GAAG*cu*T_sT	383
30434	RPI GL2 Str1 (sense) 2'-F U,C + 3' ,5' Abasic,TT; 2'-O-Me-A,G;ribo core	BcguacgcGGAAuAcuucgaTTB	384
30435	RPI GL2 Str1 (sense) 2'-F U,C + 3' ,5' Abasic,TT; 2'-deoxyA,G;ribo core	Bc*Gu*A*c*G*c*GGAAuAcuuc*GA*TTB	385
30546	RPI GL2 Str2 (antisense) 2'-F U,C 3'-dTT	ucGAAGuAuuccGcGuAcG3T	386
30551	RPI GL2 Str2 (antisense) 2'-F U,C dTddC	ucGAAGuAuuccGcGuAcGTddC	387
30557	RPI GL2 Str2 (antisense) 2'-F U,C 3'-invertedT,T	ucGAAGuAuuccGcGuAcG*T*	388
30558	RPI GL2 Str2 (antisense) 2'-F U,C 3'-invertedT,TT	ucGAAGuAuuccGcGuAcGT*T*	389
30196	FLT1:2340U21 siRNA sense iB caps w/2'FY's	B *c*AA*cc*A*c*AAAA*u*A*c*AA*c*AATT B	419
30416	FLT1:2358L21 siRNA (2340C) (antisense) TsT	*uu*G*uu*G*u*A*uuuu*G*u*GG*uu*GT$_s$T	420
29548	HBV:394L21 siRNA (414C) (antisense)	GAUGAGGCAUAGCAGCAGGTT	421
29544	HBV:414U21 siRNA pos (sense)	CCUGCUGCUAUGCCUCAUCTT	422
29556	HBV:394L21 siRNA neg (414C) (antisense) inv	GGACGACGAUACGGAGUAGTT	423
29552	HBV:414U21 siRNA pos (sense) inv	CUACUCCGUAUCGUCGUCCTT	424
30350	HBV:262U21 siRNA stab04 (sense)	B *u*GGA*cuucucuc*AA*uuuucu*A B	425
30361	HBV:280L21 siRNA (262C) (antisense) stab05	GAAAA*uu*GAGAGAAG*uccA*T$_s$T	426
30372	HBV:262U21 siRNA inv stab04 (sense)	B A*ucuuuu*AA*cucucuuc*AGG*u* B	427
30383	HBV:280L21 siRNA (262C) (antisense) inv stab05	A*ccu*GAAGAGAG*uu*AAAAGT$_s$T	428
30352	HBV:380U21 siRNA stab04 (sense)	B *u*G*u*G*ucu*G*c*GG*c*G*uuuu*A*uc*A B	429
30363	HBV:398L21 siRNA (380C) (antisense) stab05	A*u*AAAAc*G*cc*G*cAGAc*A*cAT$_s$T	430
30374	HBV:380U21 siRNA inv stab04 (sense)	B A*cu*A*uuuu*G*c*GG*c*G*ucu*G*u*G*u* B	431
30385	HBV:398L21 siRNA (380C) (antisense) inv stab05	A*c*A*c*AGAc*G*cc*G*cAAAA*u*AT$_s$T	432
30353	HBV:413U21 siRNA stab04 (sense)	B *uccu*G*cu*G*cu*A*u*G*ccuc*A*ucu* B	433

30364	HBV:431L21 siRNA (413C) (antisense) stab05	A*u*GAGG*c*A*u*AG*c*AG*c*AGGAT$_s$T	434
30375	HBV:413U21 siRNA inv stab04 (sense)	B *ucuAcuccGuAucGucGuccu* B	435
30386	HBV:431L21 siRNA (413C) (antisense) inv stab05	AGGA*c*GA*c*GA*u*A*c*GGAG*u*AT$_s$T	436
30354	HBV:462U21 siRNA stab04 (sense)	B *uAuGuuGcccGuuuGuccucu* B	437
30365	HBV:480L21 siRNA (462C) (antisense) stab05	AGGA*c*AAA*c*GGG*c*AA*c*A*u*AT$_s$T	438
30376	HBV:462U21 siRNA inv stab04 (sense)	B *ucuccuGuuuGcccGuuGuAu* B	439
30387	HBV:480L21 siRNA (462C) (antisense) inv stab05	A*u*A*c*AA*c*GGG*c*AAA*c*AGGAT$_s$T	440
30355	HBV:1580U21 siRNA stab04 (sense)	B *uGuGcAcuucGcuucAccucu* B	441
30366	HBV:1598L21 siRNA (1580C) (antisense) stab05	AGG*u*GAAG*c*GAAG*u*G*c*A*c*AT$_s$T	442
30377	HBV:1580U21 siRNA inv stab04 (sense)	B *ucuccAcuucGcuucAcGuGu* B	443
30388	HBV:1598L21 siRNA (1580C) (antisense) inv stab05	A*c*A*c*G*u*GAAG*c*GAAG*u*GGAT$_s$T	444
30356	HBV:1586U21 siRNA stab04 (sense)	B *cuucGcuucAccucuGcAcGu* B	445
30367	HBV:1604L21 siRNA (1586C) (antisense) stab05	G*u*G*c*AGAGG*u*GAAG*c*GAAGT$_s$T	446
30378	HBV:1586U21 siRNA inv stab04 (sense)	B *uGcAcGucuccAcuucGcuuc* B	447
30389	HBV:1604L21 siRNA (1586C) (antisense) inv stab05	GAAG*c*GAAG*u*GGAGA*c*G*u*GT$_s$T	448
30357	HBV:1780U21 siRNA stab04 (sense)	B AGG*cuGuAGGcAuAAAuuGGu* B	449
30368	HBV:1798L21 siRNA (1780C) (antisense) stab05	*c*AA*uuuAuGccuAcAGccu*T$_s$T	450
30379	HBV:1780U21 siRNA inv stab04 (sense)	B *uGGuuAAAuAcGGAuGucGGA* B	451
30390	HBV:1798L21 siRNA (1780C) (antisense) inv stab05	*ucc*GA*c*A*uccGuAuuuAAc*T$_s$T	452
30612	HBV:1580U21 siRNA stab07 (sense)	B *uGuGcAcuucGcuucAccu*TT B	453
30620	HBV:1598L21 siRNA (1580C) (antisense) stab08	agg*u*gaagcgaag*u*gcacaT$_s$T	454
30628	HBV:1582U21 siRNA inv stab07 (sense)	B *ucuccAcuucGcuucAcGu*TT B	455
30636	HBV:1596L21 siRNA (1578C) (antisense) inv stab08	gcacacg*u*gaagcgaag*u*gT$_s$T	456
30612	HBV:1580U21 siRNA stab07 (sense)	B *uGuGcAcuucGcuucAccu*TT B	457
31175	HBV:1598L21 siRNA (1580C) stab11 (antisense)	*AGGuGAAGcGAAGuGcAcA*T$_s$T	458
30612	HBV:1580U21 siRNA stab07 (sense)	B *uGuGcAcuucGcuucAccu*TT B	459
31176	HBV:1596L21 siRNA (1578C) (antisense) inv stab11 (antisense)	G*c*A*c*A*c*G*u*GAAG*c*GAAG*u*GT$_s$T	460
30287	HBV:1580U21 siRNA (sense)	UGUGCACUUCGCUUCACCUCU	461

30298	HBV:1598L21 siRNA (1580C) (antisense)	AGGUGAAGCGAAGUGCACACG	462
30355	HBV:1580U21 siRNA stab04 (sense)	B uGuGcAcuucGcuucAccucu B	463
30366	HBV:1598L21 siRNA (1580C) (antisense) stab05	AGGuGAAGcGAAGuGcAcATsT	464
30612	HBV:1580U21 siRNA stab07 (sense)	B uGuGcAcuucGcuucAccuTT B	465
31175	HBV:1598L21 siRNA (1580C) stab11 (antisense)	*AGGuGAAGcGAAGuGcAcA*TsT	466
30612	HBV:1580U21 siRNA stab07 (sense)	B uGuGcAcuucGcuucAccuTT B	467
30620	HBV:1598L21 siRNA (1580C) (antisense) stab08	AGGuGAAGcGAAGuGcAcATsT	468
31335	HBV:1580U21 siRNA stab09 (sense)	B UGUGCACUUCGCUUCACCUTT B	469
31337	HBV:1598L21 siRNA (1580C) stab10 (antisense)	AGGUGAAGCGAAGUGCACATsT	470

UPPER CASE = ribonucleotide
Lower case = 2'-O-methyl nucleotide
Underline = 2'-deoxy-2'-amino nucleotide
***Italic* = 2'-deoxy-2'-fluoro nucleotide**
T = thymidine
***T* = inverted thymidine**
t = 3'-deoxy thymidine
B = inverted deoxyabasic succinate linker
B = inverted deoxyabasic
X = universal base (5-nitroindole)
Z = universal base (3-nitropyrrole)
S = phosphorothioate internucleotide linkage
***U* = 5-bromodeoxyuridine**
***A* = deoxyadenosine**
***G* = deoxyguanosine**
L = glyceryl moiety
ddC = dideoxy Cytidine

Table II

A. 2.5 µmol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	6.5	163 µL	45 sec	2.5 min	7.5 min
S-Ethyl Tetrazole	23.8	238 µL	45 sec	2.5 min	7.5 min
Acetic Anhydride	100	233 µL	5 sec	5 sec	5 sec
N-Methyl Imidazole	186	233 µL	5 sec	5 sec	5 sec
TCA	176	2.3 mL	21 sec	21 sec	21 sec
Iodine	11.2	1.7 mL	45 sec	45 sec	45 sec
Beaucage	12.9	645 µL	100 sec	300 sec	300 sec
Acetonitrile	NA	6.67 mL	NA	NA	NA

B. 0.2 µmol Synthesis Cycle ABI 394 Instrument

Reagent	Equivalents	Amount	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time*RNA
Phosphoramidites	15	31 µL	45 sec	233 sec	465 sec
S-Ethyl Tetrazole	38.7	31 µL	45 sec	233 min	465 sec
Acetic Anhydride	655	124 µL	5 sec	5 sec	5 sec
N-Methyl Imidazole	1245	124 µL	5 sec	5 sec	5 sec
TCA	700	732 µL	10 sec	10 sec	10 sec
Iodine	20.6	244 µL	15 sec	15 sec	15 sec
Beaucage	7.7	232 µL	100 sec	300 sec	300 sec
Acetonitrile	NA	2.64 mL	NA	NA	NA

C. 0.2 µmol Synthesis Cycle 96 well Instrument

Reagent	Equivalents:DNA/ 2'-O-methyl/Ribo	Amount: DNA/2'-O-methyl/Ribo	Wait Time* DNA	Wait Time* 2'-O-methyl	Wait Time* Ribo
Phosphoramidites	22/33/66	40/60/120 µL	60 sec	180 sec	360sec
S-Ethyl Tetrazole	70/105/210	40/60/120 µL	60 sec	180 min	360 sec
Acetic Anhydride	265/265/265	50/50/50 µL	10 sec	10 sec	10 sec
N-Methyl Imidazole	502/502/502	50/50/50 µL	10 sec	10 sec	10 sec
TCA	238/475/475	250/500/500 µL	15 sec	15 sec	15 sec
Iodine	6.8/6.8/6.8	80/80/80 µL	30 sec	30 sec	30 sec
Beaucage	34/51/51	80/120/120	100 sec	200 sec	200 sec
Acetonitrile	NA	1150/1150/1150 µL	NA	NA	NA

- Wait time does not include contact time during delivery.

- Tandem synthesis utilizes double coupling of linker molecule

Table III

Group	Solution on Filter (1.0 µL)	Stock VEGF concentration	Number of Animals	Injectate (6.0 µL)	Dose	Conc. injectate
1	Tris-Cl pH 6.9	NA	5	water	NA	NA
2	R&D Systems VEGF-carrier free 75 µM	3.53 µg/µL	5	water	NA	NA
3	R&D Systems VEGF-carrier free 75 µM	3.53 µg/µL	5	Site 2340 Stab1 siRNA	10 µg/eye	1.67 µg/µL
4	R&D Systems VEGF-carrier free 75 µM	3.53 µg/µL	5	Site 2340 Stab1 siRNA	3 µg/eye	0.5 µg/µL
5	R&D Systems VEGF-carrier free 75 µM	3.53 µg/µL	5	Site 2340 Stab1 siRNA	1 µg/eye	0.167 µg/µL
6	R&D Systems VEGF-carrier free 75 µM	3.53 µg/µL	5	Inactive Site 2340 Stab1 siRNA	10 µg/eye	1.67 µg/µL
7	R&D Systems VEGF-carrier free 75 µM	3.53 µg/µL	5	Inactive Site 2340 Stab1 siRNA	3 µg/eye	0.5 µg/µL
8	R&D Systems VEGF-carrier free 75 µM	3.53 µg/µL	5	Inactive Site 2340 Stab1 siRNA	1 µg/eye	0.167 µg/µL

Table IV

Non-limiting examples of Stabilization Chemistries for chemically modified siNA constructs

Chemistry	pyrimidine	Purine	cap	p=S	Strand
"Stab 1"	Ribo	Ribo	-	5 at 5'-end 1 at 3'-end	S/AS
"Stab 2"	Ribo	Ribo	-	All linkages	Usually AS
"Stab 3"	2'-fluoro	Ribo	-	4 at 5'-end 4 at 3'-end	Usually S
"Stab 4"	2'-fluoro	Ribo	5' and 3'-ends	-	Usually S
"Stab 5"	2'-fluoro	Ribo	-	1 at 3'-end	Usually AS
"Stab 6"	2'-O-Methyl	Ribo	5' and 3'-ends	-	Usually S
"Stab 7"	2'-fluoro	2'-deoxy	5' and 3'-ends	-	Usually S
"Stab 8"	2'-fluoro	2'-O-Methyl	-	1 at 3'-end	Usually AS
"Stab 9"	Ribo	Ribo	5' and 3'-ends	-	Usually S
"Stab 10"	Ribo	Ribo	-	1 at 3'-end	Usually AS
"Stab 11"	2'-fluoro	2'-deoxy	-	1 at 3'-end	Usually AS

CAP = any terminal cap, see for example **Figure 10**.

All Stab 1-11 chemistries can comprise 3'-terminal thymidine (TT) residues

All Stab 1-11 chemistries typically comprise 21 nucleotides, but can vary as described herein.

S = sense strand

AS = antisense strand

CLAIMS

What we claim is:

1. A double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a target gene, wherein said siNA molecule comprises no ribonucleotides and each strand of said double-stranded siNA comprises about 21 nucleotides.

2. The siNA molecule of claim 1, wherein one of the strands of said double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of the target gene, and wherein the second strand of said double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or a portion thereof of the target gene.

3. The siNA molecule of claim 2, wherein each strand of the siNA molecule comprises about 19 to about 23 nucleotides, and wherein each strand comprises at least about 19 nucleotides that are complementary to the nucleotides of the other strand.

4. The siNA molecule of claim 1, wherein said siNA molecule comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of the target gene, and wherein said siNA further comprises a sense region, wherein said sense region comprises a nucleotide sequence substantially similar to the nucleotide sequence or a portion thereof of said target gene.

5. The siNA molecule of claim 4, wherein said antisense region and said sense region each comprise about 19 to about 23 nucleotides, and wherein said antisense region comprises at least about 19 nucleotides that are complementary to nucleotides of the sense region.

6. The siNA molecule of claim 1, wherein said siNA molecule comprises a sense region and an antisense region and wherein said antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of RNA encoded by the target gene and said sense region comprises a nucleotide sequence that is complementary to said antisense region.

7. The siNA molecule of claim 6, wherein said siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of said siNA molecule.

8. The siNA molecule of claim 6, wherein said sense region is connected to the antisense region via a linker molecule.

9. The siNA molecule of claim 8, wherein said linker molecule is a polynucleotide linker.

10. The siNA molecule of claim 8, wherein said linker molecule is a non-nucleotide linker.

11. The siNA molecule of claim 6, wherein pyrimidine nucleotides in the sense region are 2'-O-methyl pyrimidine nucleotides and purine nucleotides in the sense region are2'-deoxy purine nucleotides.

12. The siNA molecule of claim 6, wherein the pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and wherein the purine nucleotides present in the sense region are 2'-deoxy purine nucleotides.

13. The siNA molecule of claim 7, wherein the fragment comprising said sense region includes a terminal cap moiety at the 5'-end, the 3'-end, or both of the 5' and 3' ends of the fragment comprising said sense region.

14. The siNA molecule of claim 13, wherein said terminal cap moiety is an inverted deoxy abasic moiety.

15. The siNA molecule of claim 6, wherein the pyrimidine nucleotides of said antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides of said antisense region are 2'-O-methyl purine nucleotides.

16. The siNA molecule of claim 6, wherein the pyrimidine nucleotides present in said antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and wherein the purine nucleotides present in said antisense region comprise 2'-deoxy- purine nucleotides.

17. The siNA molecule of claim 15, wherein said antisense region comprises a phosphorothioate internucleotide linkage at the 3' end of said antisense region.

18. The siNA molecule of claim 6, wherein said antisense region comprises a glyceryl modification at the 3' end of said antisense region.

19. The siNA molecule of claim 7, wherein each of the two fragments of said siNA molecule comprise 21 nucleotides.

20. The siNA molecule of claim 17, wherein about 19 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule and wherein at least two 3' terminal nucleotides of each fragment of the siNA molecule are not base-paired to the nucleotides of the other fragment of the siNA molecule.

21. The siNA molecule of claim 20, wherein each of the two 3' terminal nucleotides of each fragment of the siNA molecule are 2'-deoxy-pyrimidines.

22. The siNA molecule of claim 21, wherein the 2'-deoxy-pyrimidine is 2'-deoxy-thymidine.

23. The siNA molecule of claim 19, wherein all 21 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule.

24. The siNA molecule of claim 19, wherein about 19 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the target gene.

25. The siNA molecule of claim 19, wherein 21 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the target gene.

26. The siNA molecule of claim 7, wherein the 5'-end of the fragment comprising said antisense region optionally includes a phosphate group.

27. The siNA molecule of claim 1, wherein said target gene is a mammalian gene.

28. The siNA molecule of claim 1, wherein said target gene is a plant gene.

29. The siNA molecule of claim 1, wherein said target gene is a bacterial gene.

30. The siNA molecule of claim 1, wherein said target gene is a fungal gene.

31. The siNA molecule of claim 1, wherein said target gene is a viral gene.

32. The siNA molecule of claim 27, wherein said mammalian gene is a human gene.

33. A double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a target RNA sequence, wherein said siNA molecule comprises no ribonucleotides and wherein each strand of said double-stranded siNA molecule comprises about 21 nucleotides g.

34. The siNA molecule of claim 33, wherein said target RNA sequence is encoded by a viral genome.

35. The siNA molecule of claim 33, wherein said target RNA sequence is encoded by a bacterial gene.

36. The siNA molecule of claim 33, wherein said target RNA sequence is encoded by a mammalian gene.

37. The siNA molecule of claim 36, wherein said mammalian gene is a human gene.

38. The siNA molecule of claim 33, wherein said target RNA sequence is encoded by a plant gene.

39. A double-stranded short interfering nucleic acid (siNA) molecule that inhibits the replication of a virus, wherein said siNA molecule comprises no ribonucleotides and each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

40. The siNA molecule of claim 39, wherein said virus is a mammalian virus.

41. The siNA molecule of claim 39, wherein said virus is a plant virus.

42. The siNA molecule of claim 40, wherein said mammalian virus is hepatitis C virus.

43. The siNA molecule of claim 40, wherein said mammalian virus is human immunodeficiency virus.

44. The siNA molecule of claim 40, wherein said mammalian virus is hepatitis B virus.

45. The siNA molecule of claim 40, wherein said mammalian virus is herpes simplex virus.

46. The siNA molecule of claim 40, wherein said mammalian virus is cytomegalovirus.

47. The siNA molecule of claim 40, wherein said mammalian virus is human papilloma virus.

48. The siNA molecule of claim 40, wherein said mammalian virus is respiratory syncytial virus.

49. The siNA molecule of claim 40, wherein said mammalian virus is influenza virus.

50. A double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a target gene, wherein said siNA molecule does not require the presence of a ribonucleotide within the siNA molecule for said inhibition of expression of a target gene and wherein each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

51. The siNA molecule of claim 50, wherein said target gene is a mammalian gene.

52. The siNA molecule of claim 50, wherein said target gene is a plant gene.

53. The siNA molecule of claim 50, wherein said target gene is a bacterial gene.

54. The siNA molecule of claim 50, wherein said target gene is a fungal gene.

55. The siNA molecule of claim 50, wherein said target gene is a viral gene.

56. The siNA molecule of claim 51, wherein said mammalian gene is a human gene.

57. A double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a target gene by mediating RNA interference (RNAi) process, wherein said siNA molecule comprises no ribonucleotides and wherein each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

58. The siNA molecule of claim 57, wherein said target gene is encoded by a viral genome.

59. The siNA molecule of claim 57, wherein said target gene is a bacterial gene.

60. The siNA molecule of claim 57, wherein said target gene is a mammalian gene.

61. The siNA molecule of claim 60, wherein said mammalian gene is a human gene.

62. The siNA molecule of claim 57, wherein said target gene is a plant gene.

63. A double-stranded short interfering nucleic acid (siNA) molecule that inhibits the replication of a virus, wherein said siNA molecule does not require the presence of a ribonucleotide within the siNA molecule for said inhibition of replication of a virus and each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

64. The siNA molecule of claim 63, wherein said virus is a mammalian virus.

65. The siNA molecule of claim 63, wherein said virus is a plant virus.

66. The siNA molecule of claim 64, wherein said mammalian virus is hepatitis C virus.

67. The siNA molecule of claim 64, wherein said mammalian virus is human immunodeficiency virus.

68. The siNA molecule of claim 64, wherein said mammalian virus is hepatitis B virus.

69. The siNA molecule of claim 64, wherein said mammalian virus is herpes simplex virus.

70. The siNA molecule of claim 64, wherein said mammalian virus is cytomegalovirus.

71. The siNA molecule of claim 64, wherein said mammalian virus is human papilloma virus.

72. The siNA molecule of claim 64, wherein said mammalian virus is respiratory syncytial virus.

73. The siNA molecule of claim 64, wherein said mammalian virus is influenza virus.

74. A pharmaceutical composition comprising the siNA molecule of any of claims 1, 33, 39, 50, 57 and 63 in an acceptable carrier or diluent.

75. Medicament comprising a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a target gene, wherein said siNA molecule comprises no ribonucleotides and each strand of said double-stranded siNA comprises about 21 nucleotides.

76. Medicament according to claim 75, wherein one of the strands of said double-stranded siNA molecule comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of the target gene, and wherein the second strand of said double-stranded siNA molecule comprises a nucleotide sequence substantially similar to the nucleotide sequence or a portion thereof of the target gene.

77. Medicament according to claim 76, wherein each said strand of the siNA molecule comprises about 19 to about 23 nucleotides, and wherein each said strand comprises at least about 19 nucleotides that are complementary to the nucleotides of the other strand.

78. Medicament according to claim 75, wherein said siNA molecule comprises an antisense region comprising a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of the target gene, and wherein said siNA further comprises a sense region, wherein said sense region comprises a nucleotide sequence substantially similar to the nucleotide sequence or a portion thereof of said target gene.

79. Medicament according to claim 78, wherein said antisense region and said sense region each comprise about 19 to about 23 nucleotides, and wherein said antisense region comprises at least about 19 nucleotides that are complementary to nucleotides of the sense region.

80. Medicament according to claim 75, wherein said siNA molecule comprises a sense region and an antisense region and wherein said antisense region comprises a nucleotide sequence that is complementary to a nucleotide sequence or a portion thereof of RNA encoded by the target gene and said sense region comprises a nucleotide sequence that is complementary to said antisense region.

81. Medicament according to claim 80, wherein said siNA molecule is assembled from two separate oligonucleotide fragments wherein one fragment comprises the sense region and the second fragment comprises the antisense region of said siNA molecule.

82. Medicament according to claim 80, wherein said sense region is connected to the antisense region via a linker molecule.

83. Medicament according to claim 82, wherein said linker molecule is a polynucleotide linker.

84. Medicament according to claim 82, wherein said linker molecule is a non-nucleotide linker.

85. Medicament according to claim 80, wherein pyrimidine nucleotides in the sense region are 2'-O-methyl pyrimidine nucleotides and purine nucleotides in the sense region are2'-deoxy purine nucleotides.

86. Medicament according to claim 80, wherein the pyrimidine nucleotides present in the sense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and wherein the purine nucleotides present in the sense region are 2'-deoxy purine nucleotides.

87. Medicament according to claim 81, wherein the fragment comprising said sense region includes a terminal cap moiety at the 5'-end, the 3'-end, or both of the 5' and 3' ends of the fragment comprising said sense region.

88. Medicament according to claim 87, wherein said terminal cap moiety is an inverted deoxy abasic moiety.

89. Medicament according to claim 80, wherein the pyrimidine nucleotides of said antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and the purine nucleotides of said antisense region are 2'-O-methyl purine nucleotides.

90. Medicament according to claim 80, wherein the pyrimidine nucleotides present in said antisense region are 2'-deoxy-2'-fluoro pyrimidine nucleotides and wherein the purine nucleotides present in said antisense region comprise 2'-deoxy- purine nucleotides.

91. Medicament according to claim 89, wherein said antisense region comprises a phosphorothioate internucleotide linkage at the 3' end of said antisense region.

92. Medicament according to claim 80, wherein said antisense region comprises a glyceryl modification at the 3' end of said antisense region.

93. Medicament according to 81, wherein each of the two fragments of said siNA molecule comprise 21 nucleotides.

94. Medicament according to claim 91, wherein about 19 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule and wherein at least two 3' terminal nucleotides of each fragment of the siNA molecule are not base-paired to the nucleotides of the other fragment of the siNA molecule.

95. Medicament according to claim 94, wherein each of the two 3' terminal nucleotides of each fragment of the siNA molecule are 2'-deoxy-pyrimidines.

96. Medicament according to claim 95, wherein said 2'-deoxy-pyrimidine is 2'-deoxy-thymidine.

97. Medicament according to claim 93, wherein all 21 nucleotides of each fragment of the siNA molecule are base-paired to the complementary nucleotides of the other fragment of the siNA molecule.

98. Medicament according to claim 93, wherein about 19 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the target gene.

99. Medicament according to claim 93, wherein 21 nucleotides of the antisense region are base-paired to the nucleotide sequence or a portion thereof of the RNA encoded by the target gene.

100. Medicament according to claim 81, wherein the 5'-end of the fragment comprising said antisense region optionally includes a phosphate group.

101. Medicament according to claim 75, wherein said target gene is a mammalian gene.

102. Medicament according to claim 75, wherein said target gene is a plant gene.

103. Medicament according to claim 75, wherein said target gene is a bacterial gene.

104. Medicament according to claim 75, wherein said target gene is a fungal gene.

105. Medicament according to claim 75, wherein said target gene is a viral gene.

106. Medicament according to claim 101, wherein said mammalian gene is a human gene.

107. Medicament comprising a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a target RNA sequence, wherein said siNA molecule comprises no ribonucleotides and wherein each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

108. Medicament according to claim 107, wherein said target RNA sequence is encoded by a viral genome.

109. Medicament according to claim 107, wherein said target RNA sequence is encoded by a bacterial gene.

110. Medicament according to claim 107, wherein said target RNA sequence is encoded by a mammalian gene.

111. Medicament according to claim 110, wherein said mammalian gene is a human gene.

112. Medicament according to claim 107, wherein said target RNA sequence is encoded by a plant gene.

113. Medicament comprising a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the replication of a virus, wherein said siNA molecule comprises no ribonucleotides and each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

114. Medicament according to claim 113, wherein said virus is a mammalian virus.

115. Medicament according to claim 113, wherein said virus is a plant virus.

116. Medicament according to claim 114, wherein said mammalian virus is hepatitis C virus.

117. Medicament according to claim 114, wherein said mammalian virus is human immunodeficiency virus.

118. Medicament according to claim 114, wherein said mammalian virus is hepatitis B virus.

119. Medicament according to claim 114, wherein said mammalian virus is herpes simplex virus.

120. Medicament according to claim 114, wherein said mammalian virus is cytomegalovirus.

121. Medicament according to claim 114, wherein said mammalian virus is human papilloma virus.

122. Medicament according to claim 114, wherein said mammalian virus is respiratory syncytial virus.

123. Medicament according to claim 114, wherein said mammalian virus is influenza virus.

124. Medicament comprising a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a target gene, wherein said siNA molecule does not require the presence of a ribonucleotide within the siNA molecule for said inhibition of expression of a target gene and wherein each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

125. Medicament according to claim 124, wherein said target gene is a mammalian gene.

126. Medicament according to claim 124, wherein said target gene is a plant gene.

127. Medicament according to claim 124, wherein said target gene is a bacterial gene.

128. Medicament according to claim 124, wherein said target gene is a fungal gene.

129. Medicament according to claim 124, wherein said target gene is a viral gene.

130. Medicament according to claim 125, wherein said mammalian gene is a human gene.

131. Medicament comprising a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a target gene by mediating RNA interference (RNAi) process, wherein said siNA molecule comprises no ribonucleotides and wherein each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

132. Medicament comprising a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the replication of a virus, wherein said siNA molecule does not require the presence of a ribonucleotide within the siNA molecule for said inhibition of replication of a virus and each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

133. Medicament according to claim 132, wherein said target gene is encoded by a viral genome.

134. Medicament according to claim 132, wherein said target gene is a bacterial gene.

135. Medicament according to claim 132, wherein said target gene is a mammalian gene.

136. Medicament according to claim 135, wherein said mammalian gene is a human gene.

137. Medicament according to claim 132, wherein said target gene is a plant gene.

138. Active ingredient comprising a double-stranded short interfering nucleic acid (siNA) molecule that down-regulates expression of a target gene, wherein said siNA molecule comprises no ribonucleotides and each strand of said double-stranded siNA comprises about 21 nucleotides.

139. Active ingredient comprising a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a target RNA sequence, wherein said siNA molecule comprises no ribonucleotides and wherein each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

140. Active ingredient comprising a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the replication of a virus, wherein said siNA molecule comprises no ribonucleotides and each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

141. Active ingredient comprising a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a target gene, wherein said siNA molecule does not require the presence of a ribonucleotide within the siNA molecule for said inhibition of expression of a target gene and wherein each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

142. Active ingredient comprising a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the expression of a target gene by mediating RNA interference (RNAi) process, wherein said siNA molecule comprises no ribonucleotides and wherein each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

143. Active ingredient comprising a double-stranded short interfering nucleic acid (siNA) molecule that inhibits the replication of a virus, wherein said siNA molecule does not require the presence of a ribonucleotide within the siNA molecule for said inhibition of replication of a virus and each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

144. Use of a double-stranded short interfering nucleic acid (siNA) molecule to down-regulate expression of a target gene, wherein said siNA molecule comprises no ribonucleotides and each strand of said double-stranded siNA comprises about 21 nucleotides.

145. Use of a double-stranded short interfering nucleic acid (siNA) molecule to inhibit the expression of a target RNA sequence, wherein said siNA molecule comprises no ribonucleotides and wherein each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

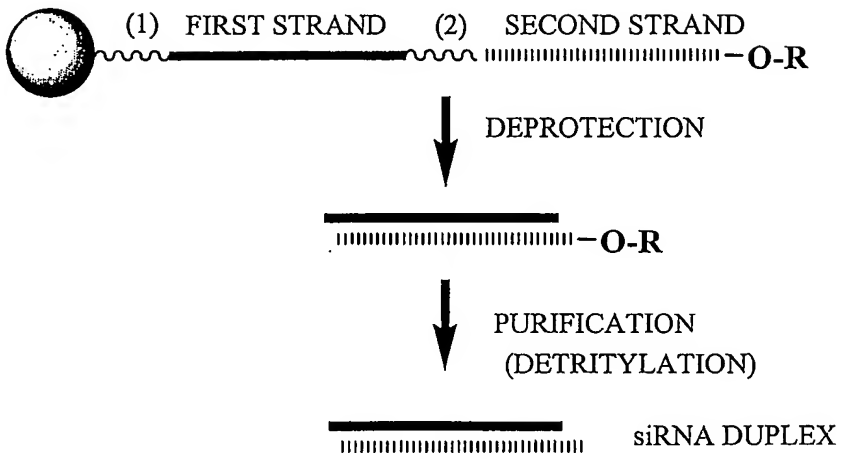
146. Use of a double-stranded short interfering nucleic acid (siNA) molecule to inhibit the replication of a virus, wherein said siNA molecule comprises no ribonucleotides and each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

147. Use of a double-stranded short interfering nucleic acid (siNA) molecule to inhibit the expression of a target gene, wherein said siNA molecule does not require the presence of a ribonucleotide within the siNA molecule for said inhibition of expression of a target gene and wherein each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

148. Use of a double-stranded short interfering nucleic acid (siNA) molecule to inhibit the expression of a target gene by mediating RNA interference (RNAi) process, wherein said siNA molecule comprises no ribonucleotides and wherein each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

149. Use of a double-stranded short interfering nucleic acid (siNA) molecule to inhibit the replication of a virus, wherein said siNA molecule does not require the presence of a ribonucleotide within the siNA molecule for said inhibition of replication of a virus and each strand of said double-stranded siNA molecule comprises about 21 nucleotides.

Figure 1

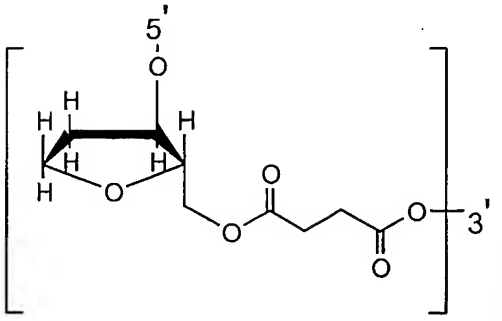


= SOLID SUPPORT

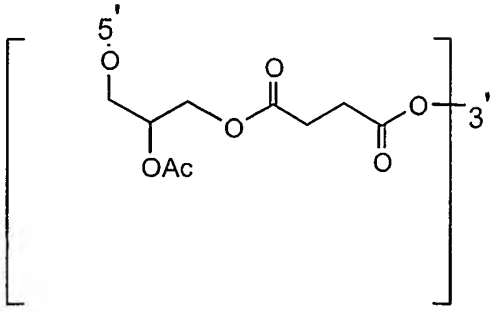
R = TERMINAL PROTECTING GROUP
FOR EXAMPLE:
DIMETHOXYTRITYL (DMT)

(1) = CLEAVABLE LINKER
(FOR EXAMPLE: NUCLEOTIDE SUCCINATE OR INVERTED DEOXYABASIC SUCCINATE)

(2) = CLEAVABLE LINKER
(FOR EXAMPLE: NUCLEOTIDE SUCCINATE OR INVERTED DEOXYABASIC SUCCINATE)



INVERTED DEOXYABASIC SUCCINATE LINKAGE



GLYCERYL SUCCINATE LINKAGE

Figure 2

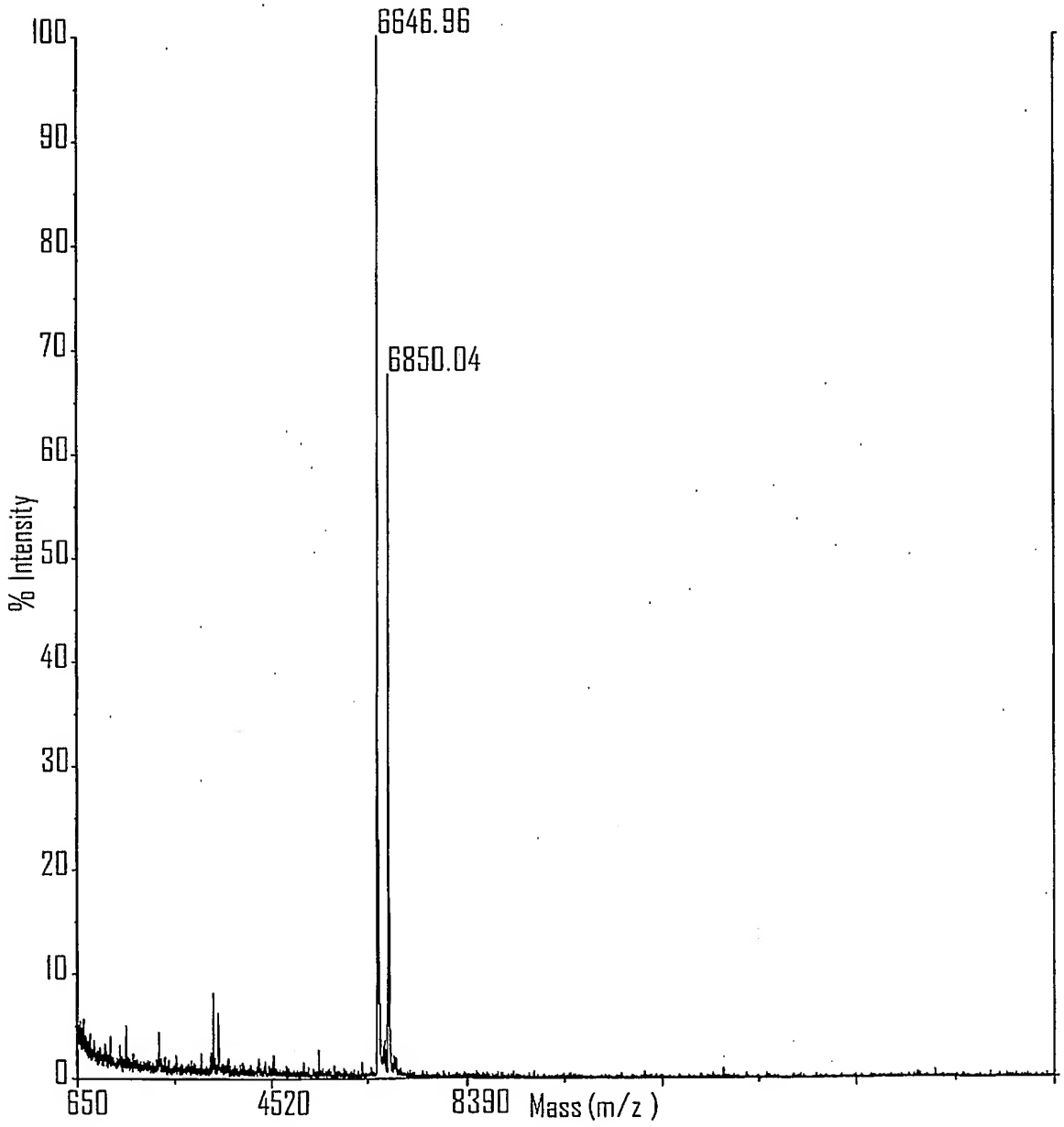

6646.96
6850.04
100
90
80
70
60
50
40
30
20
10
0
% Intensity
650
4520
8390
Mass (m/z)

Figure 3

5'-CGUACGCGGAAUACUUCGATT (SEQ ID NO: 493)
3'-TTGCAUGCGCCUUAUGAAGCU (SEQ ID NO: 494) T ½ = 15 seconds (control)

5'-B cAAccAcAAAAuAcAAcAATT B (SEQ ID NO: 493)
3'-TXGuuGGuGuuuuAuGuuGuu (SEQ ID NO: 495) T ½ = 138 min

5'-B cAAccAcAAAAuAcAAcAATT B (SEQ ID NO: 493)
3'-TDGuuGGuGuuuuAuGuuGuu (SEQ ID NO: 496) T ½ = 3.7 days

5'-B cAAccAcAAAAuAcAAcAATT B (SEQ ID NO: 493)
3'-XTGuuGGuGuuuuAuGuuGuu (SEQ ID NO: 497) T ½ = 72 minutes

5'-B cAAccAcAAAAuAcAAcAATT B (SEQ ID NO: 493)
3'-LTGuuGGuGuuuuAuGuuGuu (SEQ ID NO: 498) T ½ = 40 days

5'-B cAAccAcAAAAuAcAAcAATT B (SEQ ID NO: 493)
3'-tTGuuGGuGuuuuAuGuuGuu (SEQ ID NO: 499) T ½ = 32 days

G, A, U, C = Guanosine, Adenosine, Uridine, Cytidine
T = Thymidine
Lower Case = 2'-deoxy-2'-fluoro
S = phosphorothioate
B = inverted deoxyabasic
G = terminal glycine
D = inverted Thymidine
X = 3'-deoxy Thymidine
t = L-thymidine
L = Glyceryl moiety

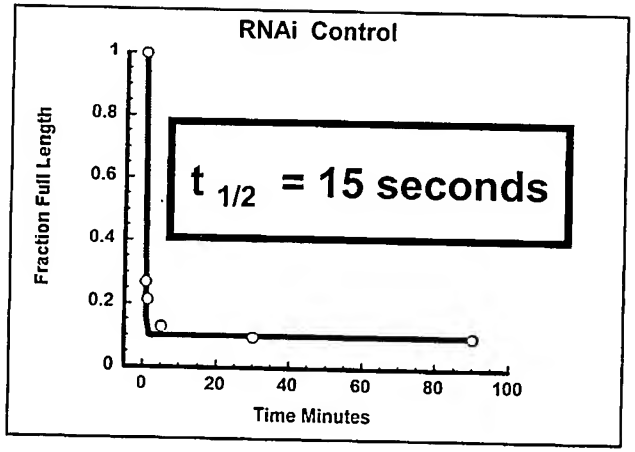


Figure 4

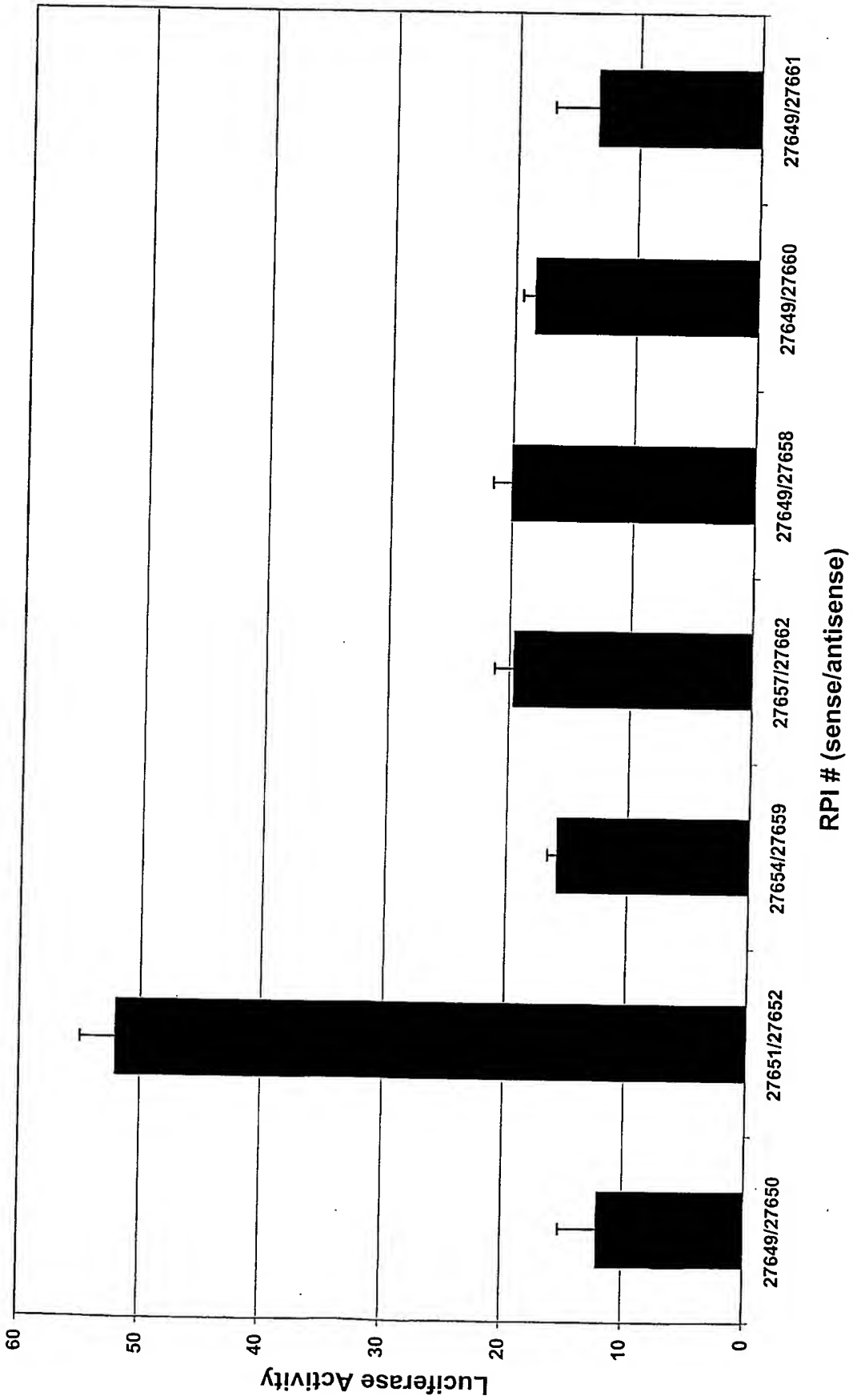

Luciferase Activity
60
50
40
30
20
10
0
27649/27650
27651/27652
27654/27659
27657/27662
27649/27658
27649/27660
27649/27661
RPI # (sense/antisense)

Figure 5

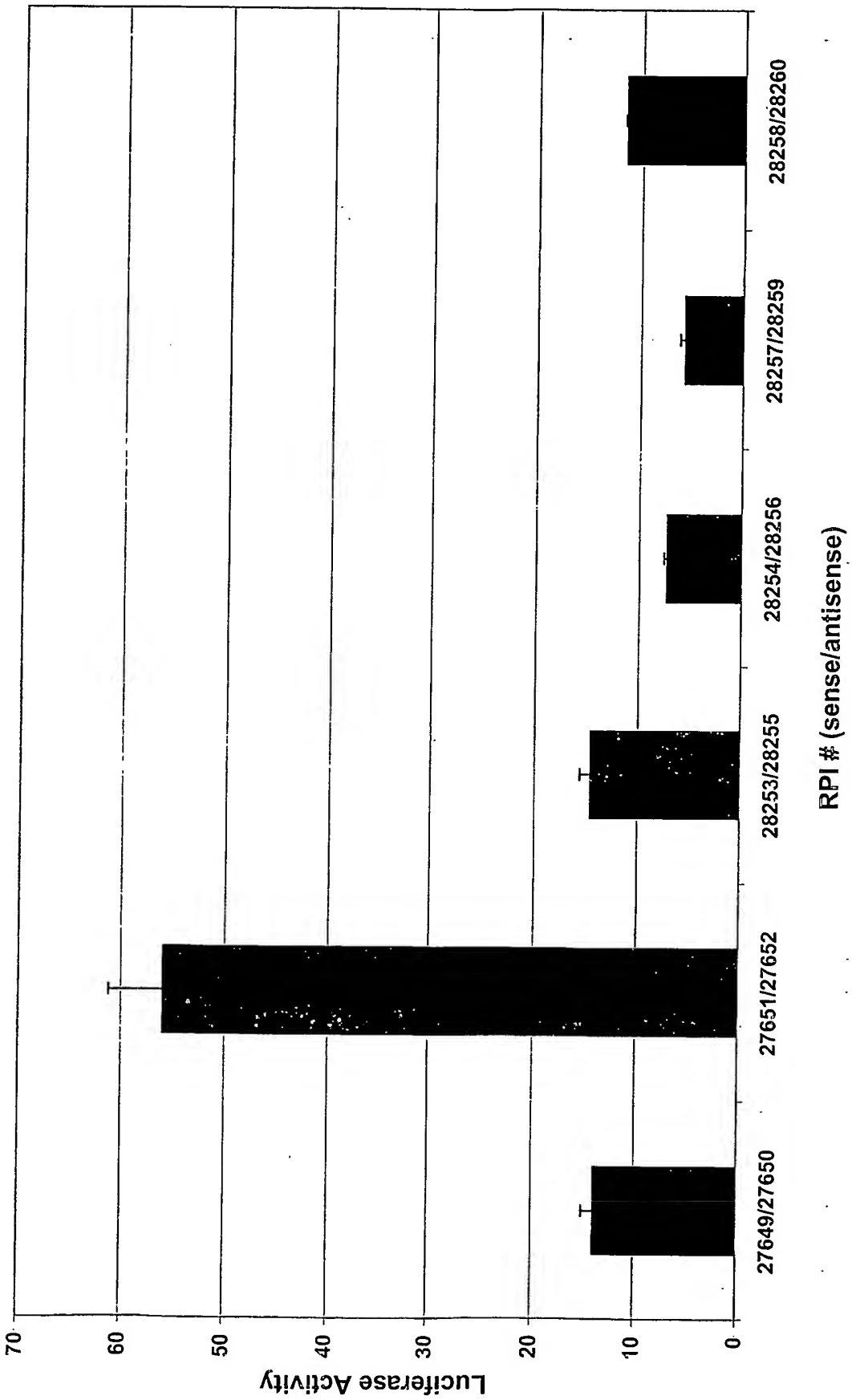

Luciferase Activity
70
60
50
40
30
20
10
0
27649/27650
27651/27652
28253/28255
28254/28256
28257/28259
28258/28260
RPI # (sense/antisense)

Figure 6

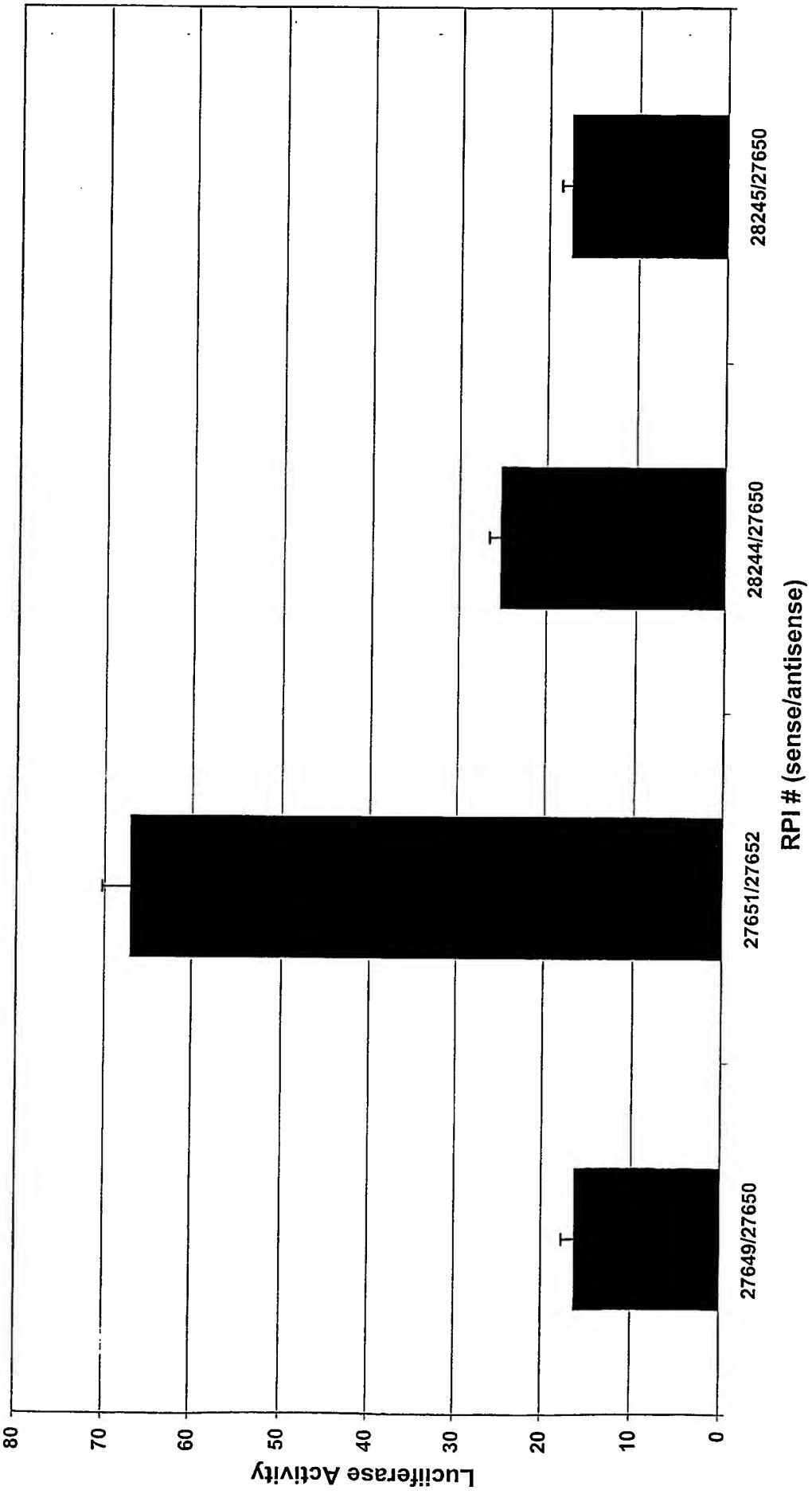
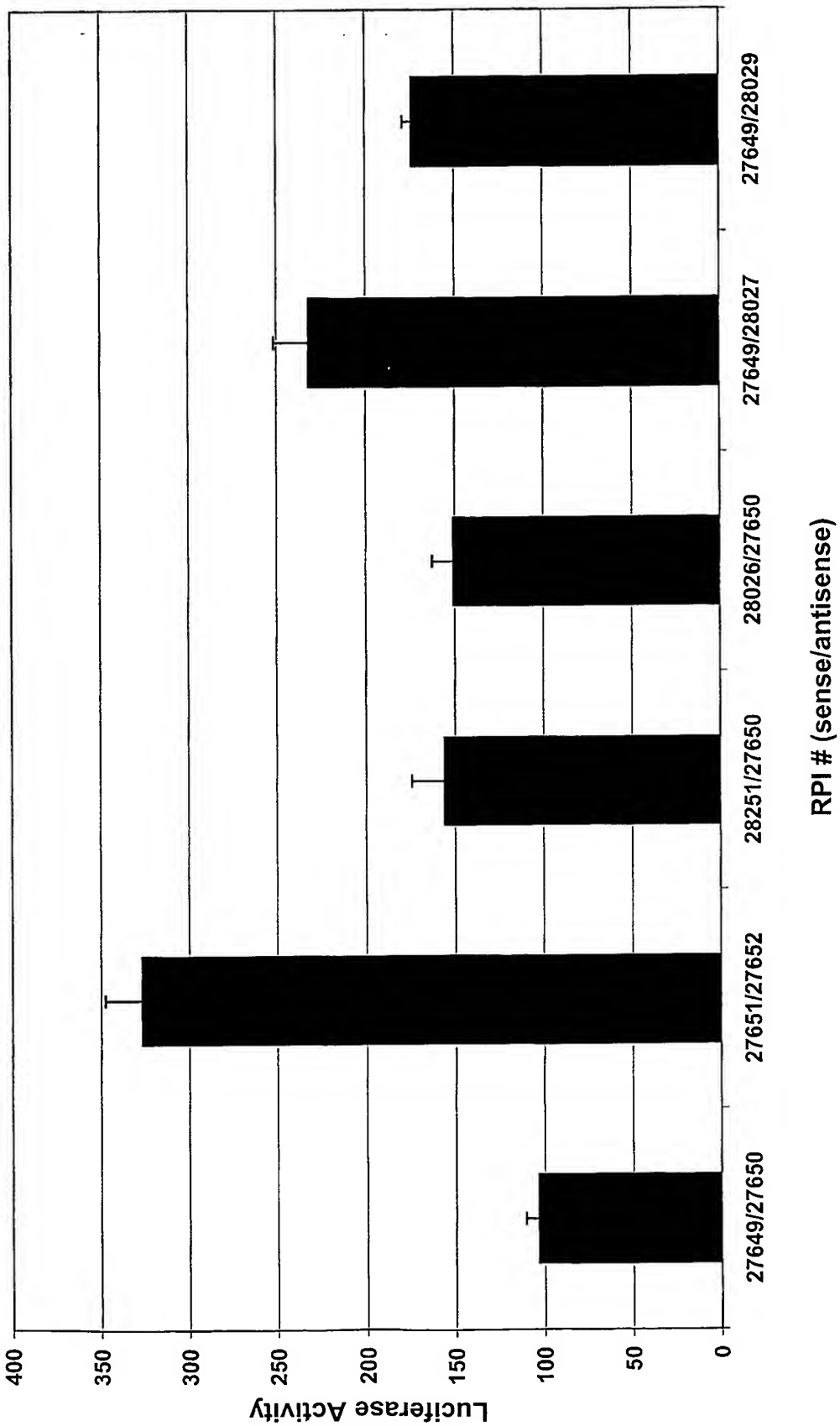

Luciferase Activity
80
70
60
50
40
30
20
10
0
27649/27650
27651/27652
28244/27650
28245/27650
RPI # (sense/antisense)

Figure 7


Luciferase Activity
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350
300
250
200
150
100
50
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27649/27650
27651/27652
28251/27650
28026/27650
27649/28027
27649/28029
RPI # (sense/antisense)

Figure 8

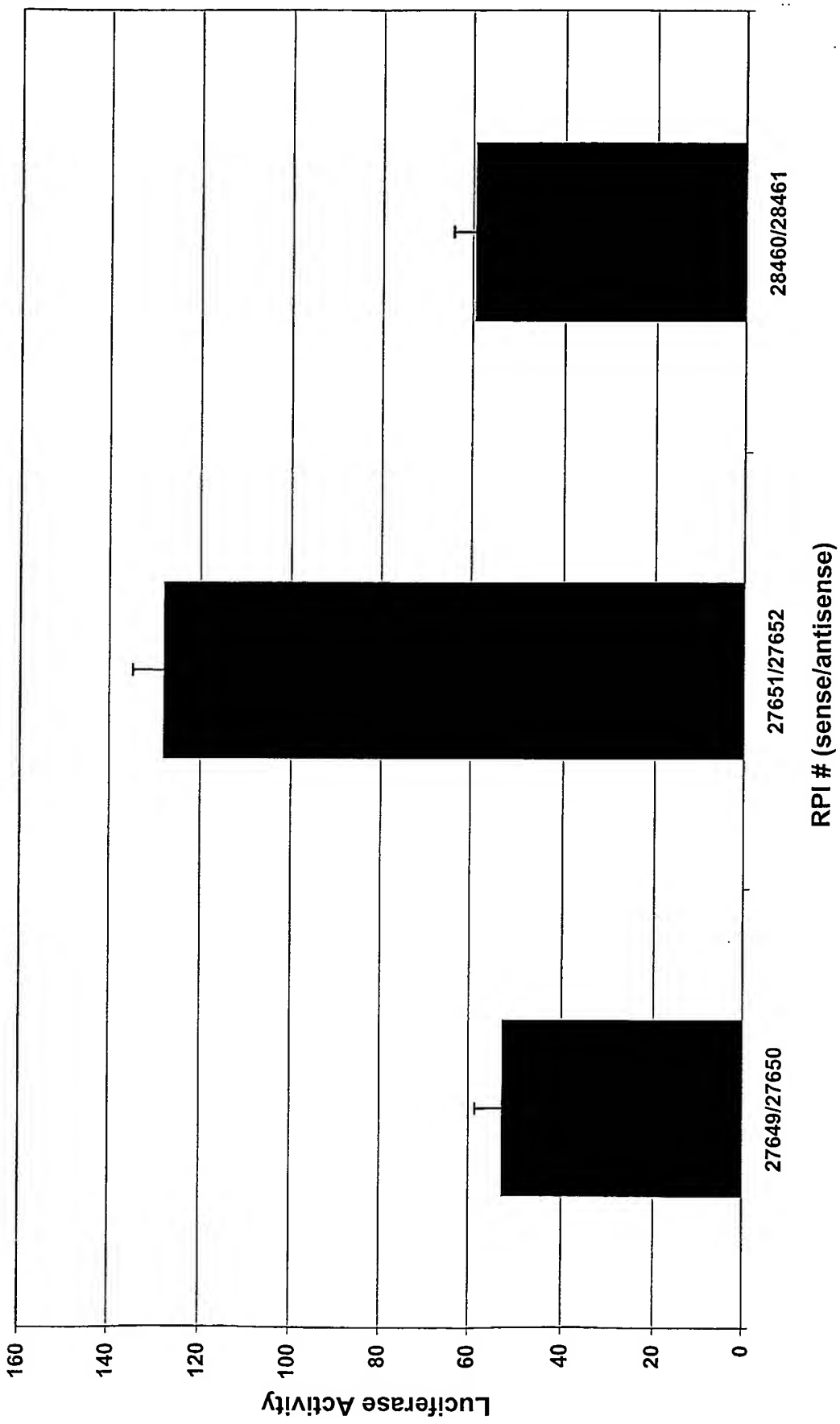
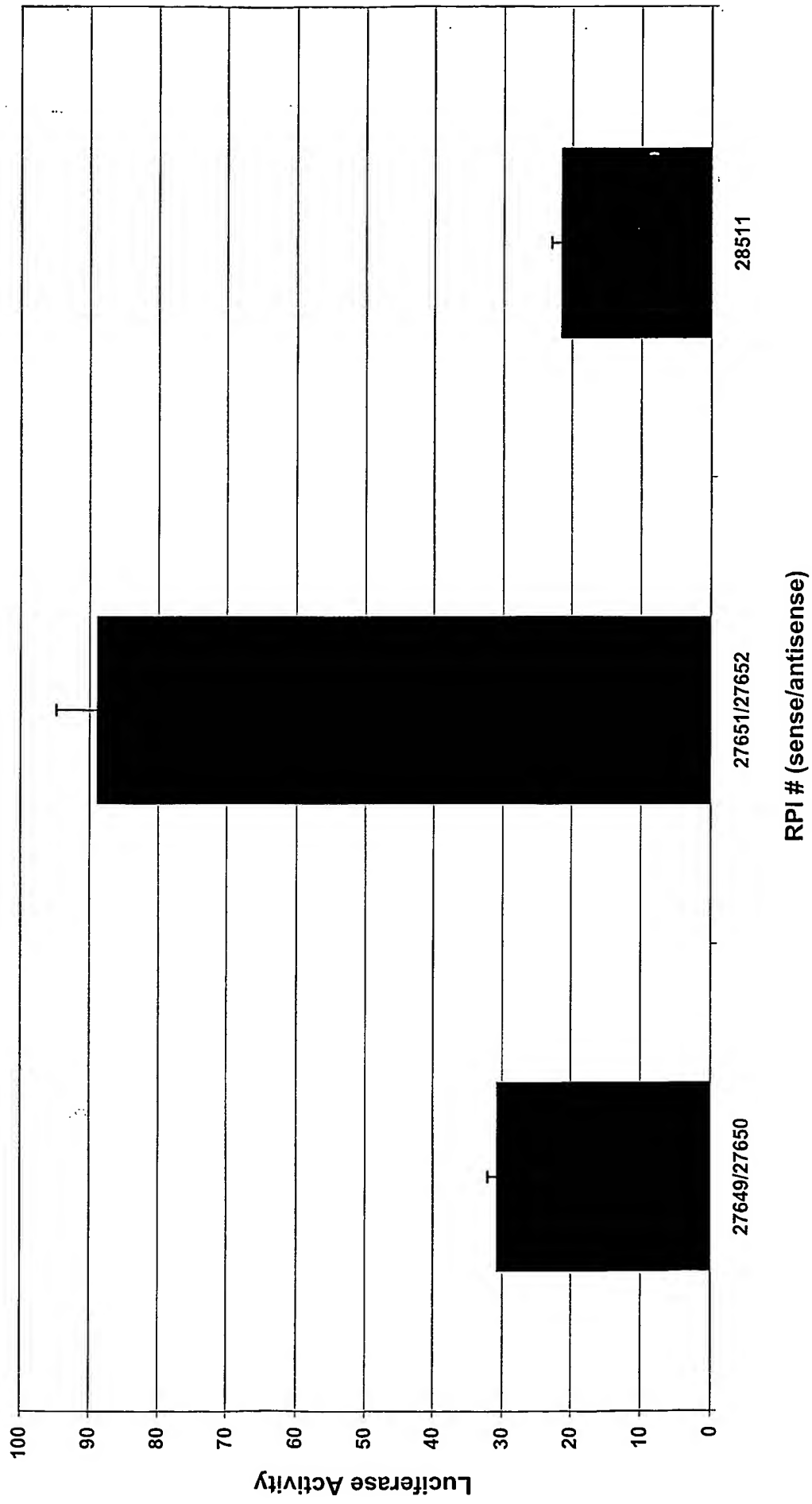

Luciferase Activity
160
140
120
100
80
60
40
20
0
27649/27650
27651/27652
28460/28461
RPI # (sense/antisense)

Figure 9


Luciferase Activity
100
90
80
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40
30
20
10
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27649/27650
27651/27652
28511
RPI # (sense/antisense)

Figure 10

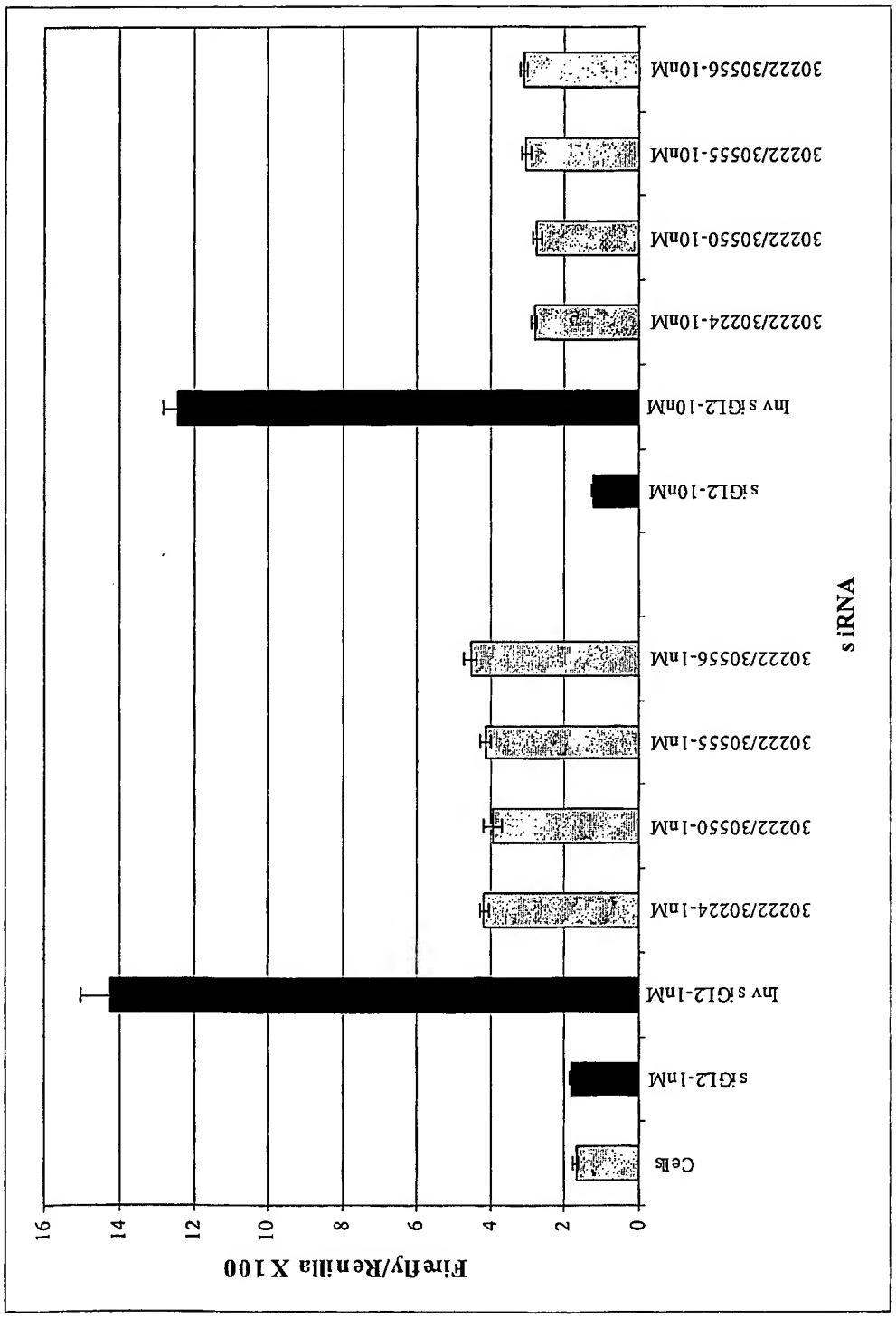
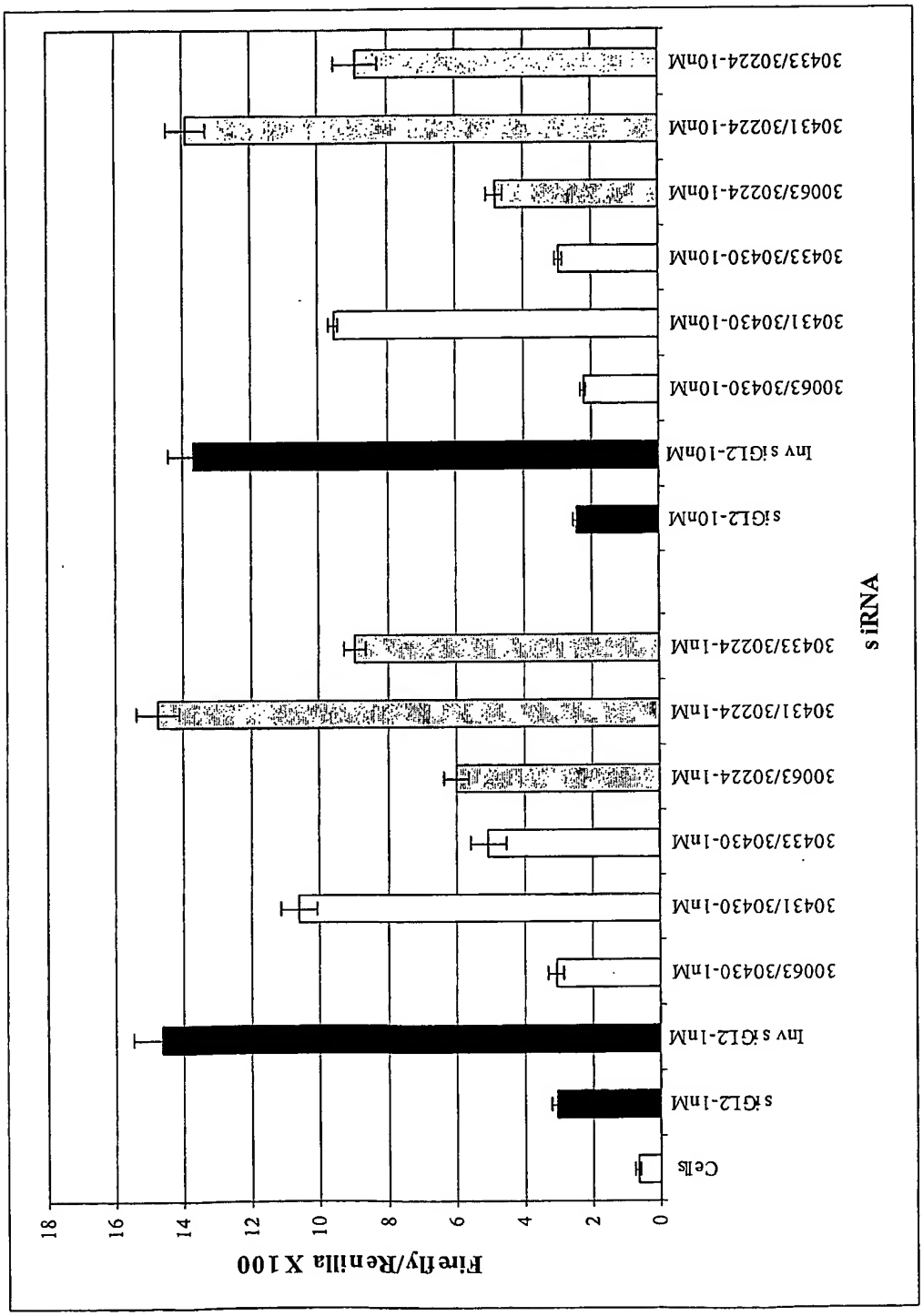

Cells
siGL2-1nM
Inv siGL2-1nM
30222/30224-1nM
30222/30550-1nM
30222/30555-1nM
30222/30556-1nM
siGL2-10nM
Inv siGL2-10nM
30222/30224-10nM
30222/30550-10nM
30222/30555-10nM
30222/30556-10nM
siRNA
Fire fly/Renilla X 100
0
2
4
6
8
10
12
14
16

Figure 11


Firefly/Renilla X 100
0
2
4
6
8
10
12
14
16
18
Cells
siGL2-1nM
Inv siGL2-1nM
30063/30430-1nM
30431/30430-1nM
30433/30430-1nM
30063/30224-1nM
30431/30224-1nM
30433/30224-1nM
siGL2-10nM
Inv siGL2-10nM
30063/30430-10nM
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30433/30430-10nM
30063/30224-10nM
30431/30224-10nM
30433/30224-10nM
siRNA

Figure 12

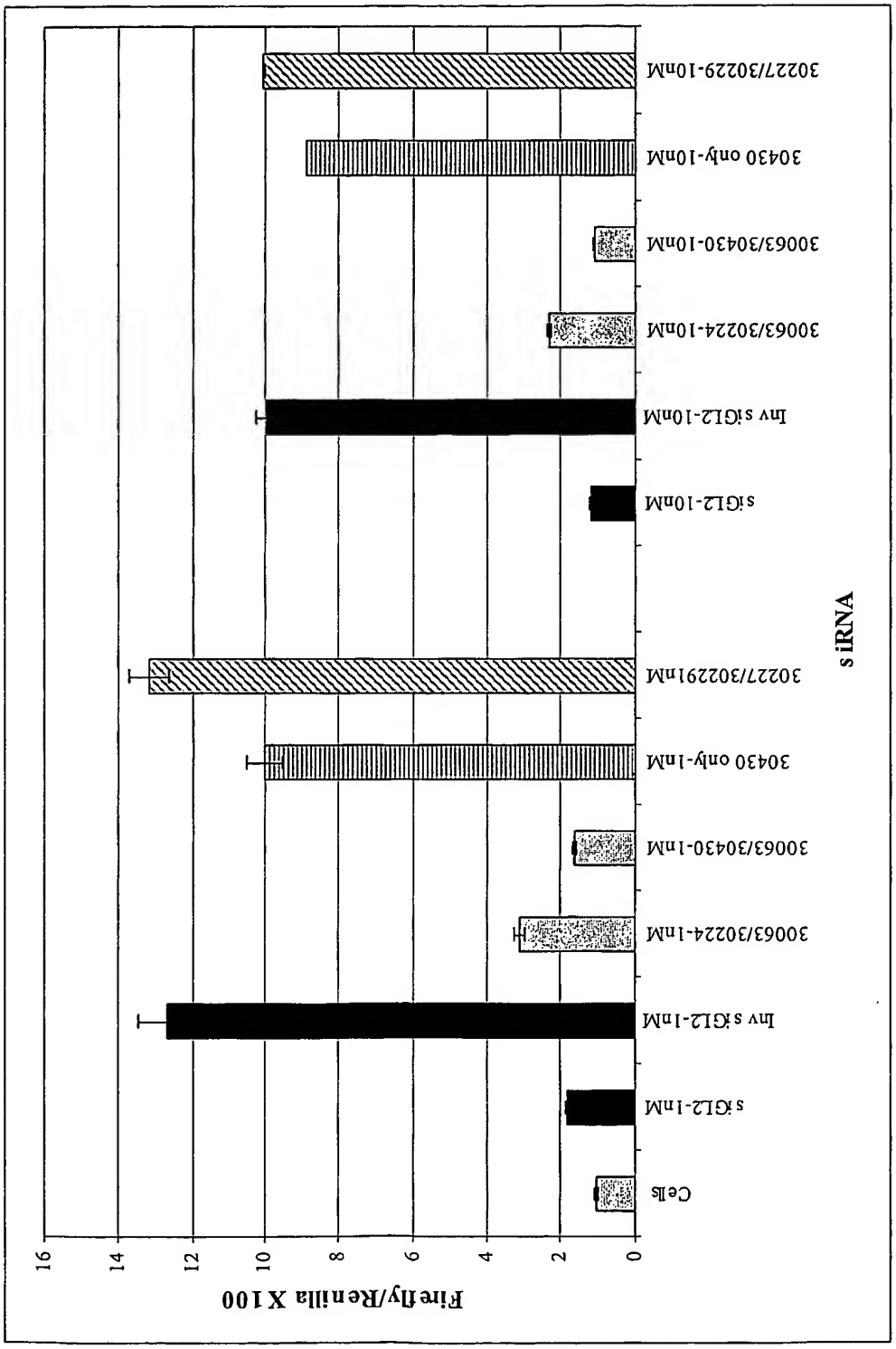

Fire fly/Renilla X 100
0
2
4
6
8
10
12
14
16
siRNA
Cells
siGL2-1nM
Inv siGL2-1nM
30063/30224-1nM
30063/30430-1nM
30430 only-1nM
30227/30229 1nM
siGL2-10nM
Inv siGL2-10nM
30063/30224-10nM
30063/30430-10nM
30430 only-10nM
30227/30229-10nM

Figure 13

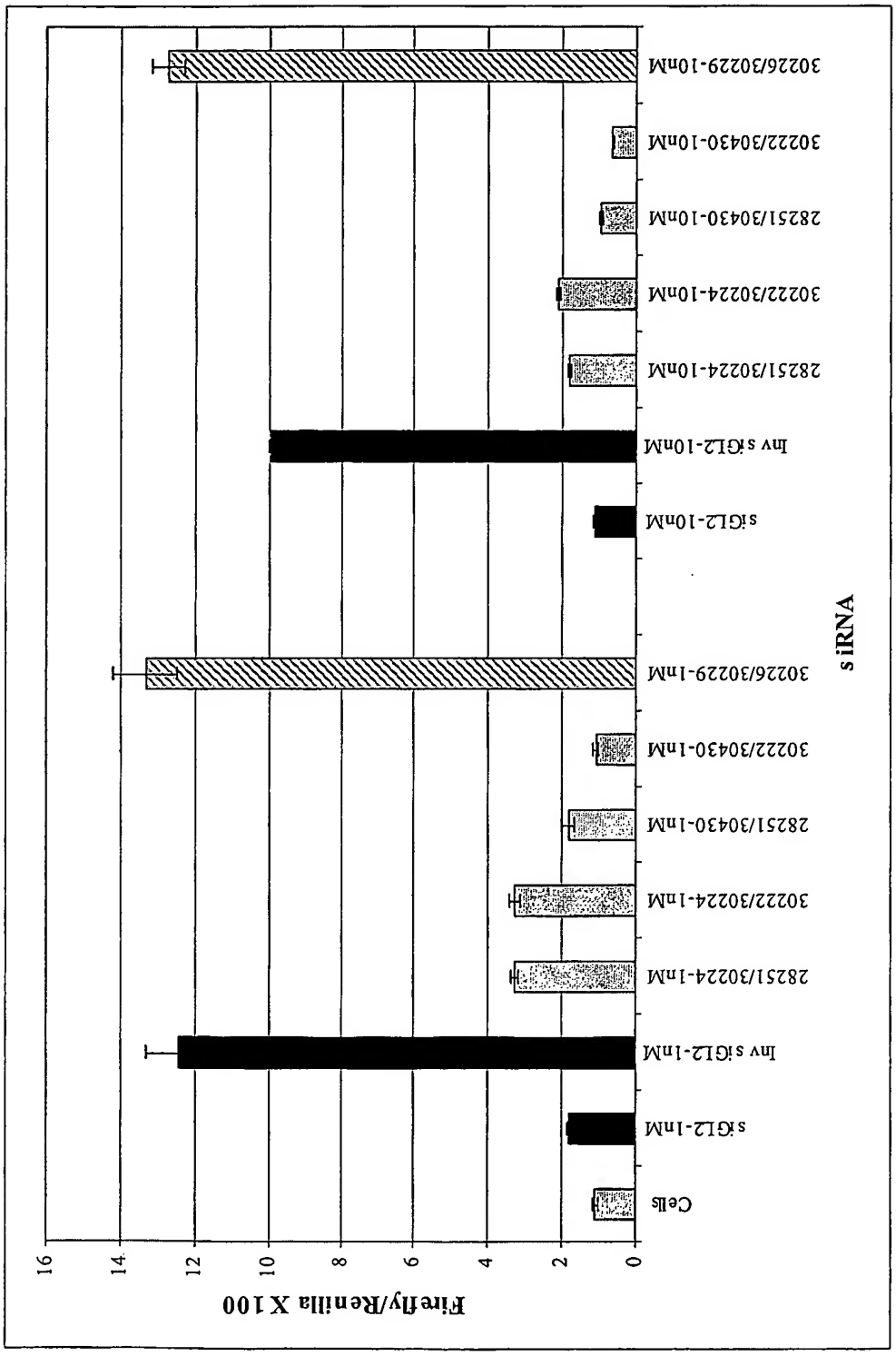

Cells
siGL2-1nM
Inv siGL2-1nM
28251/30224-1nM
30222/30224-1nM
28251/30430-1nM
30222/30430-1nM
30226/30229-1nM
siGL2-10nM
Inv siGL2-10nM
28251/30224-10nM
30222/30224-10nM
28251/30430-10nM
30222/30430-10nM
30226/30229-10nM
siRNA
FireFly/Renilla X 100
0
2
4
6
8
10
12
14
16

Figure 14

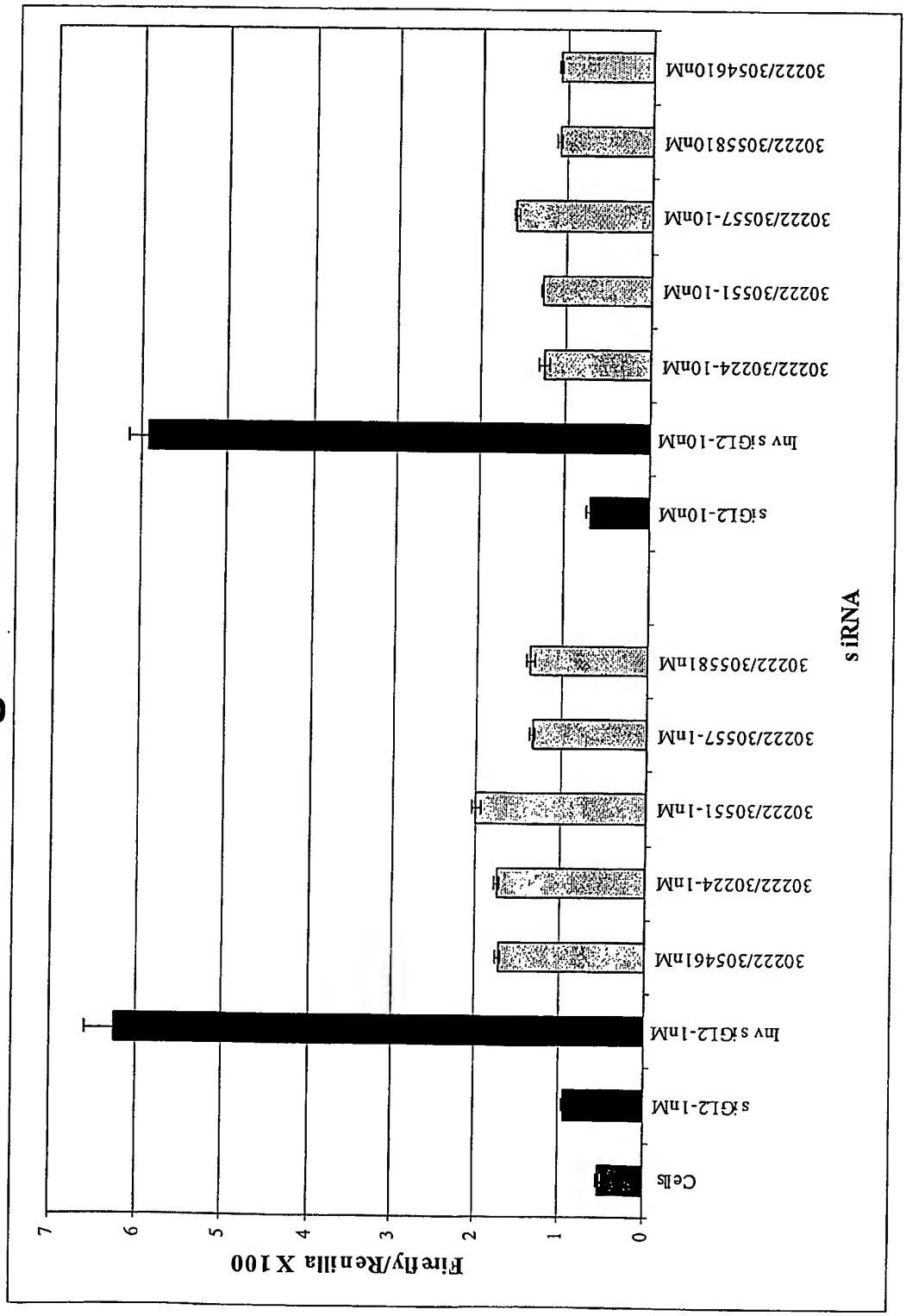

Cells
siGL2-1nM
Inv siGL2-1nM
30222/30546 1nM
30222/30224-1nM
30222/30551-1nM
30222/30557-1nM
30222/30558 1nM
siGL2-10nM
Inv siGL2-10nM
30222/30224-10nM
30222/30551-10nM
30222/30557-10nM
30222/30558 10nM
30222/30546 10nM
siRNA
Firefly/Renilla X 100
0
1
2
3
4
5
6
7

Figure 15

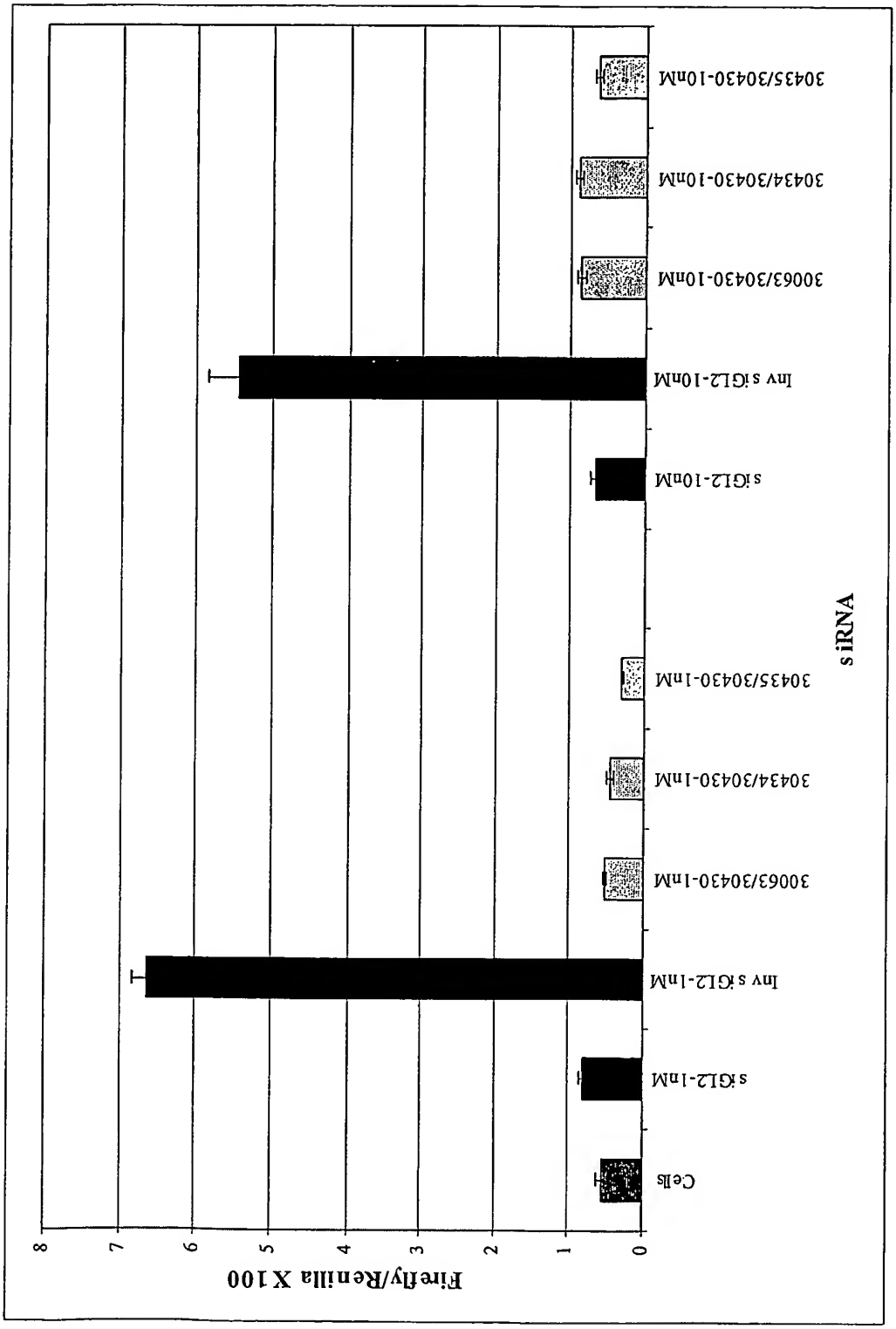

FireFly/Renilla X 100
0
1
2
3
4
5
6
7
8
Cells
siGL2-1nM
Inv siGL2-1nM
30063/30430-1nM
30434/30430-1nM
30435/30430-1nM
siGL2-10nM
Inv siGL2-10nM
30063/30430-10nM
30434/30430-10nM
30435/30430-10nM
siRNA

Figure 16

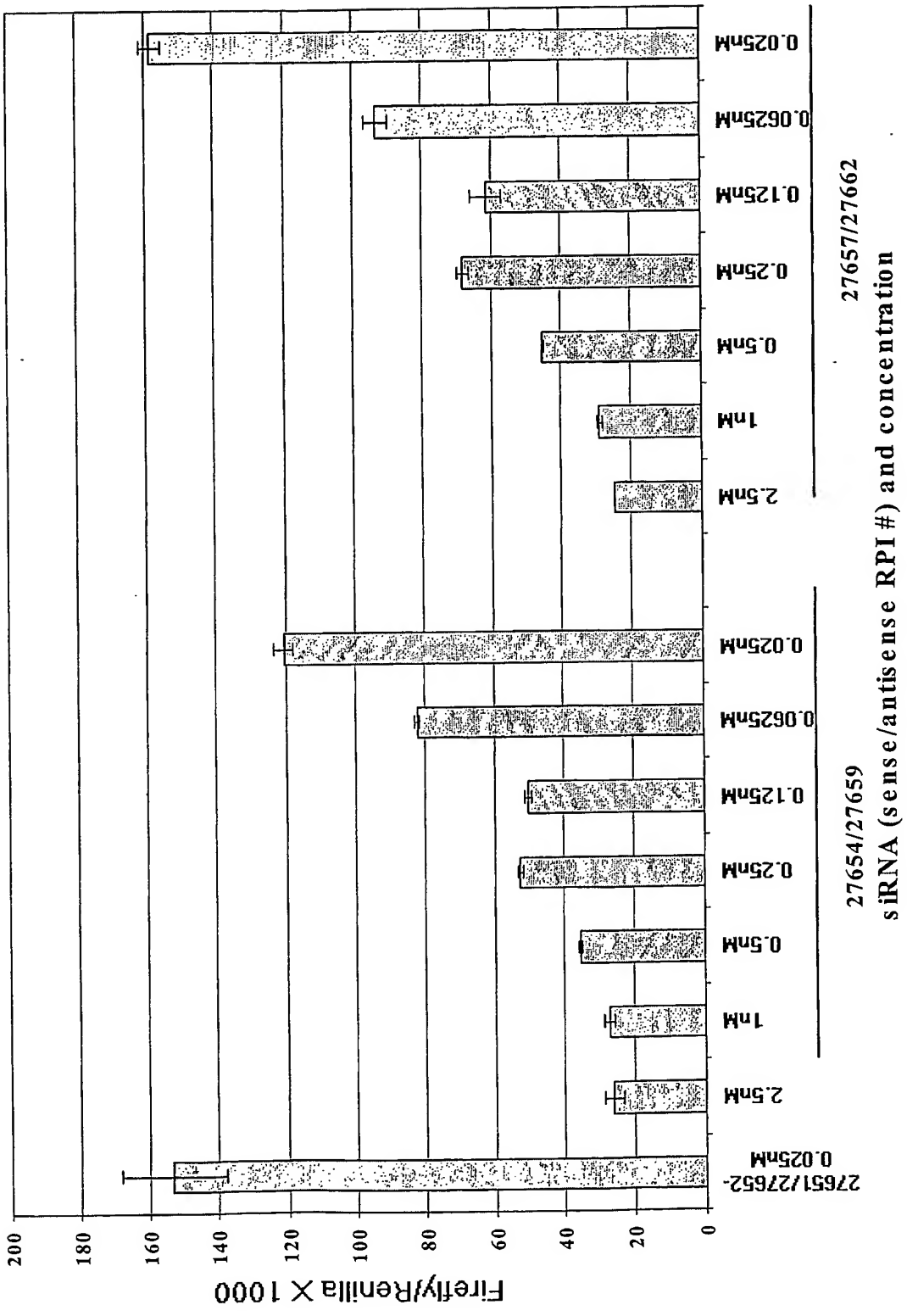

Firefly/Renilla X 1000
200
180
160
140
120
100
80
60
40
20
0
27651/27652-
0.025nM
2.5nM
1nM
0.5nM
0.25nM
0.125nM
0.0625nM
0.025nM
27654/27659
2.5nM
1nM
0.5nM
0.25nM
0.125nM
0.0625nM
0.025nM
27657/27662
siRNA (sense/antisense RPI #) and concentration

Figure 17

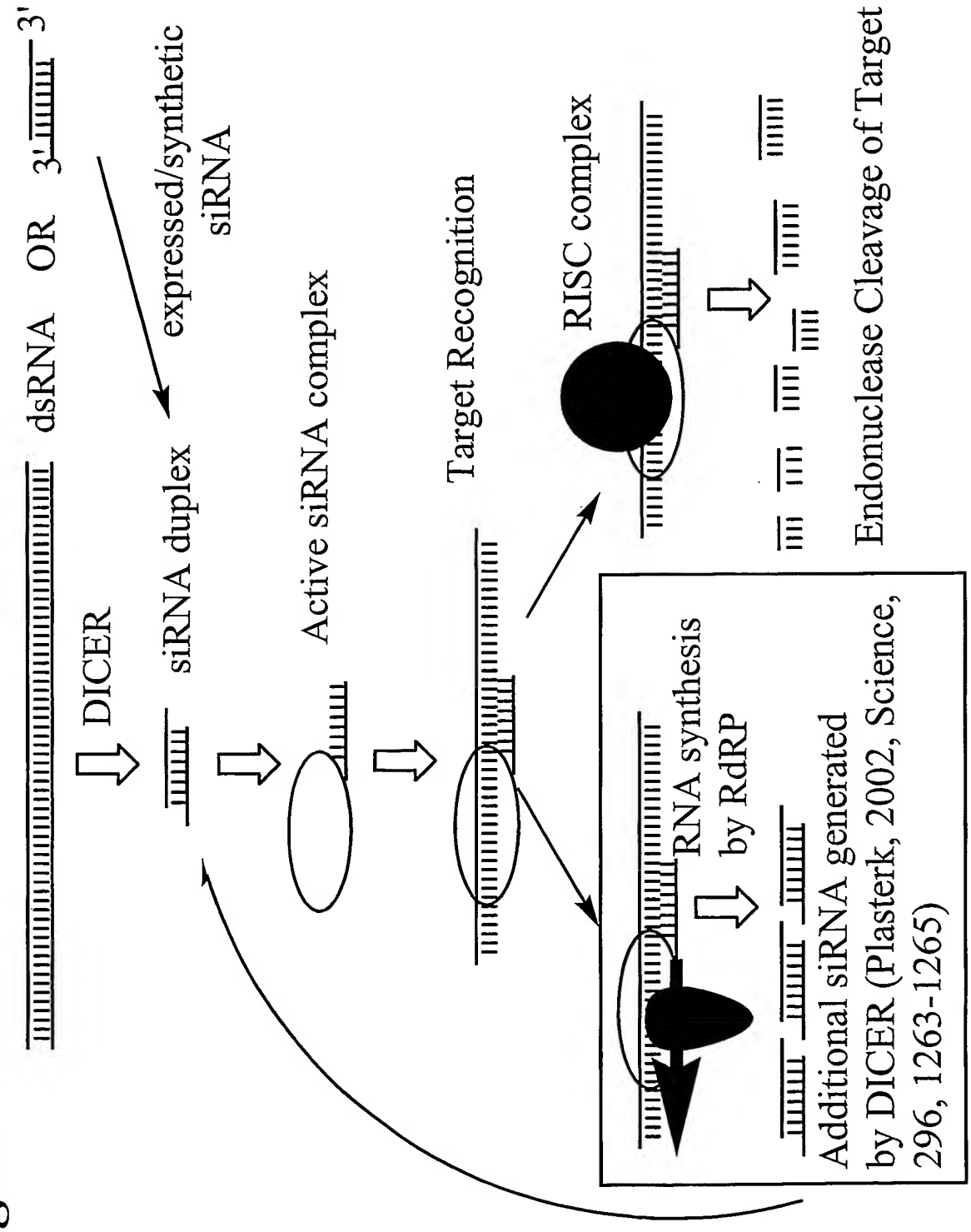

dsRNA OR 3' 3'
DICER
siRNA duplex
expressed/synthetic siRNA
Active siRNA complex
Target Recognition
RISC complex
RNA synthesis by RdRP
Additional siRNA generated by DICER (Plasterk, 2002, Science, 296, 1263-1265)
Endonuclease Cleavage of Target

Figure 18

A

SENSE STRAND (SEQ ID NO 471)
ALL PYRIMIDINES = 2'-O-ME OR 2'-FLUORO EXCEPT POSITIONS (N N)

5'- $N_S N_S N_S N_S N N N N N N N N N N N N N N N_S N_S N_S (N_S N)$ -3'

3'- L-$(N_S N)$ N N N N N N N N N N N N N N N N $N_S N_S N_S N_S N$ -5'

ANTISENSE STRAND (SEQ ID NO 472)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N)

B

SENSE STRAND (SEQ ID NO 473)
ALL PYRIMIDINES = 2'-O-ME OR 2'-FLUORO EXCEPT POSITIONS (N N)

5'- N N N N N N N N N N N N N N N N N N N N (N N) -3'

3'- L-(N N) N N N N N N N N N N N N N N N N N N N N N -5'

ANTISENSE STRAND (SEQ ID NO 474)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N)

C

SENSE STRAND (SEQ ID NO 475)
ALL PYRIMIDINES = 2'-O-ME OR 2'-FLUORO EXCEPT POSITIONS (N N)

5'- B-N N N N N N N N N N N N N N N N N N N N (N N)-B -3'

3'- L-$(N_S N)$ N N N N N N N N N N N N N N N N N N N N N -5'

ANTISENSE STRAND (SEQ ID NO 476)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N)

D

SENSE STRAND (SEQ ID NO 477)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N) AND ALL PURINES = 2'-DEOXY

5'- B-N N N N N N N N N N N N N N N N N N N N (N N)-B -3'

3'- L-$(N_S N)$ N N N N N N N N N N N N N N N N N N N N -5'

ANTISENSE STRAND (SEQ ID NO 478)
ALL PYRIMIDINES = 2'-FLUORO AND ALL PURINES = 2'-O-ME EXCEPT POSITIONS (N N)

E

SENSE STRAND (SEQ ID NO 479)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N)

5'- B-N N N N N N N N N N N N N N N N N N N N (N N)-B -3'

3'- L-(N N) N N N N N N N N N N N N N N N N N N N N -5'

ANTISENSE STRAND (SEQ ID NO 480)
ALL PYRIMIDINES = 2'-FLUORO AND ALL PURINES = 2'-O-ME EXCEPT POSITIONS (N N)

F

SENSE STRAND (SEQ ID NO 477)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N) AND ALL PURINES = 2'-DEOXY

5'- B-N N N N N N N N N N N N N N N N N N N N (N N)-B -3'

3'- L-$(N_S N)$ N N N N N N N N N N N N N N N N N N N N -5'

ANTISENSE STRAND (SEQ ID NO 481)
ALL PYRIMIDINES = 2'-FLUORO EXCEPT POSITIONS (N N) AND ALL PURINES = 2'-DEOXY

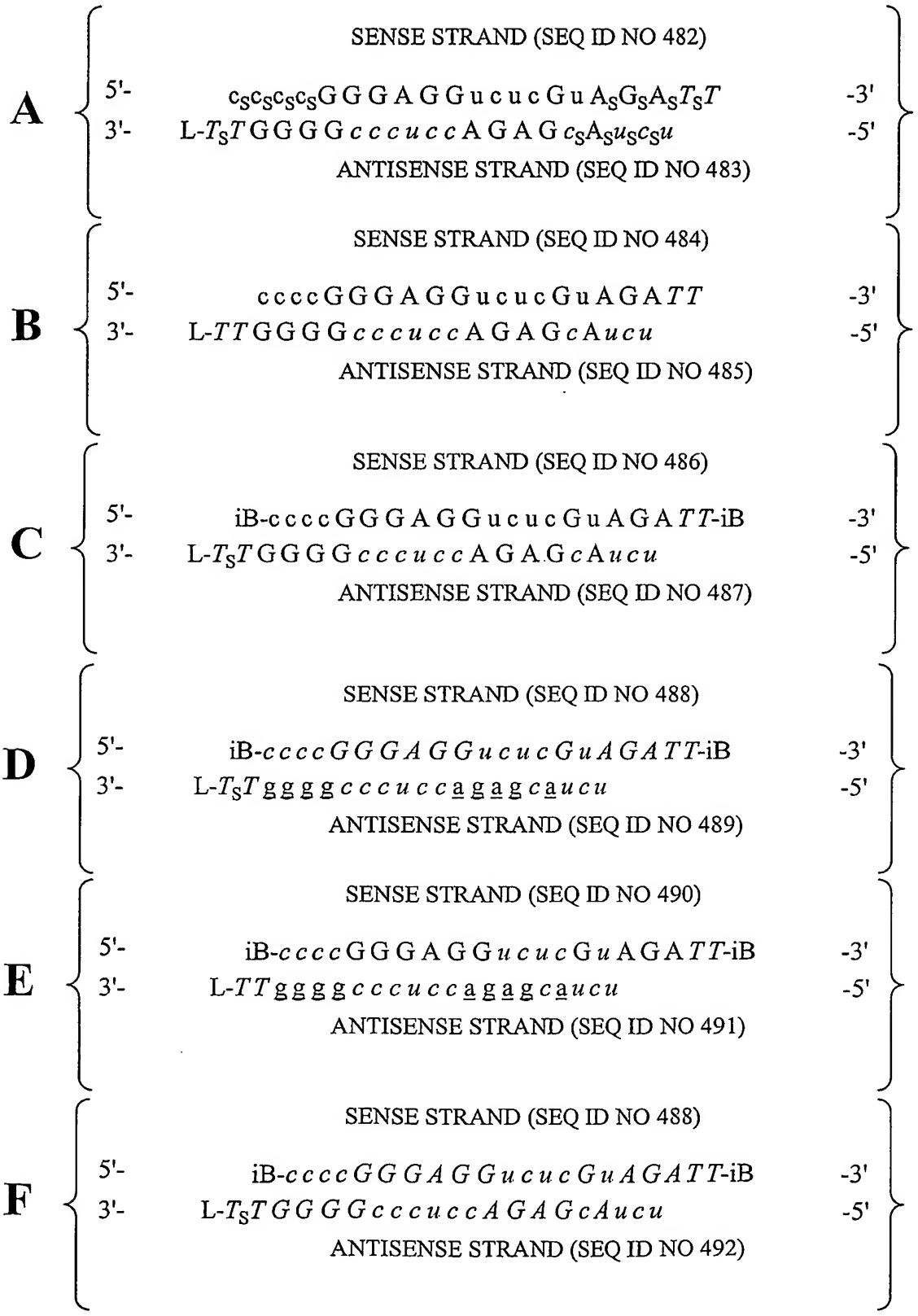
POSITIONS (NN) CAN COMPRISE ANY NUCLEOTIDE, SUCH AS DEOXYNUCLEOTIDES (eg. THYMIDINE) OR UNIVERSAL BASES

B = ABASIC, INVERTED ABASIC, INVERTED NUCLEOTIDE OR OTHER TERMINAL CAP THAT IS OPTIONALLY PRESENT

L = GLYCERYL MOIETY THAT IS OPTIONALLY PRESENT

S = PHOSPHOROTHIOATE OR PHOSPHORODITHIOATE

Figure 19



lower case = 2'-O-Methyl or 2'-deoxy-2'-fluoro
italic lower case = 2'-deoxy-2'-fluoro
underline = 2'-O-methyl

ITALIC UPPER CASE = DEOXY
B = INVERTED DEOXYABASIC
L = GLYCERYL MOIETY OPTIONALLY PRESENT
S = PHOSPHOROTHIOATE OR PHOSPHORODITHIOATE

Figure 20

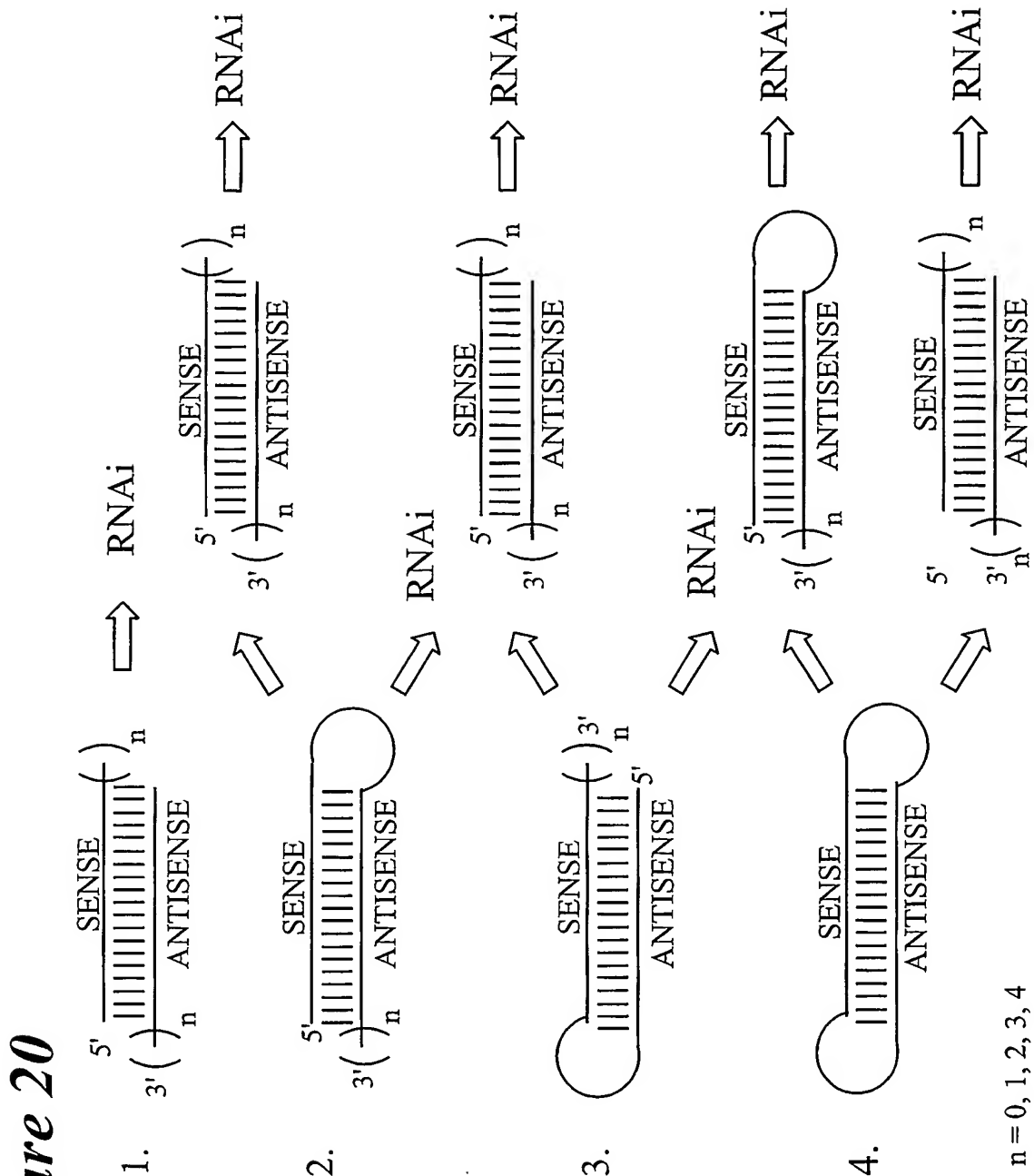


Figure 21: Target site Selection using siRNA

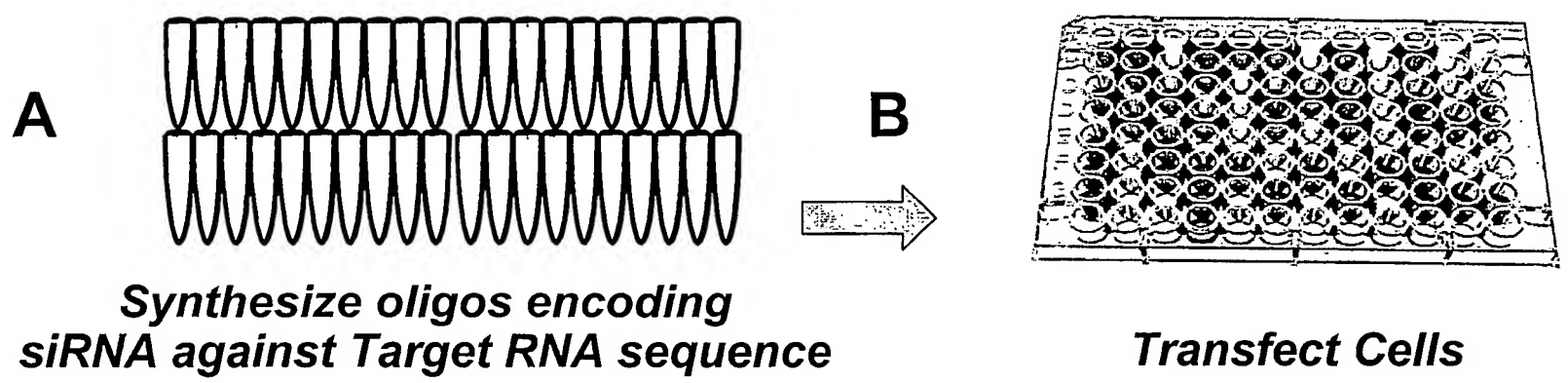
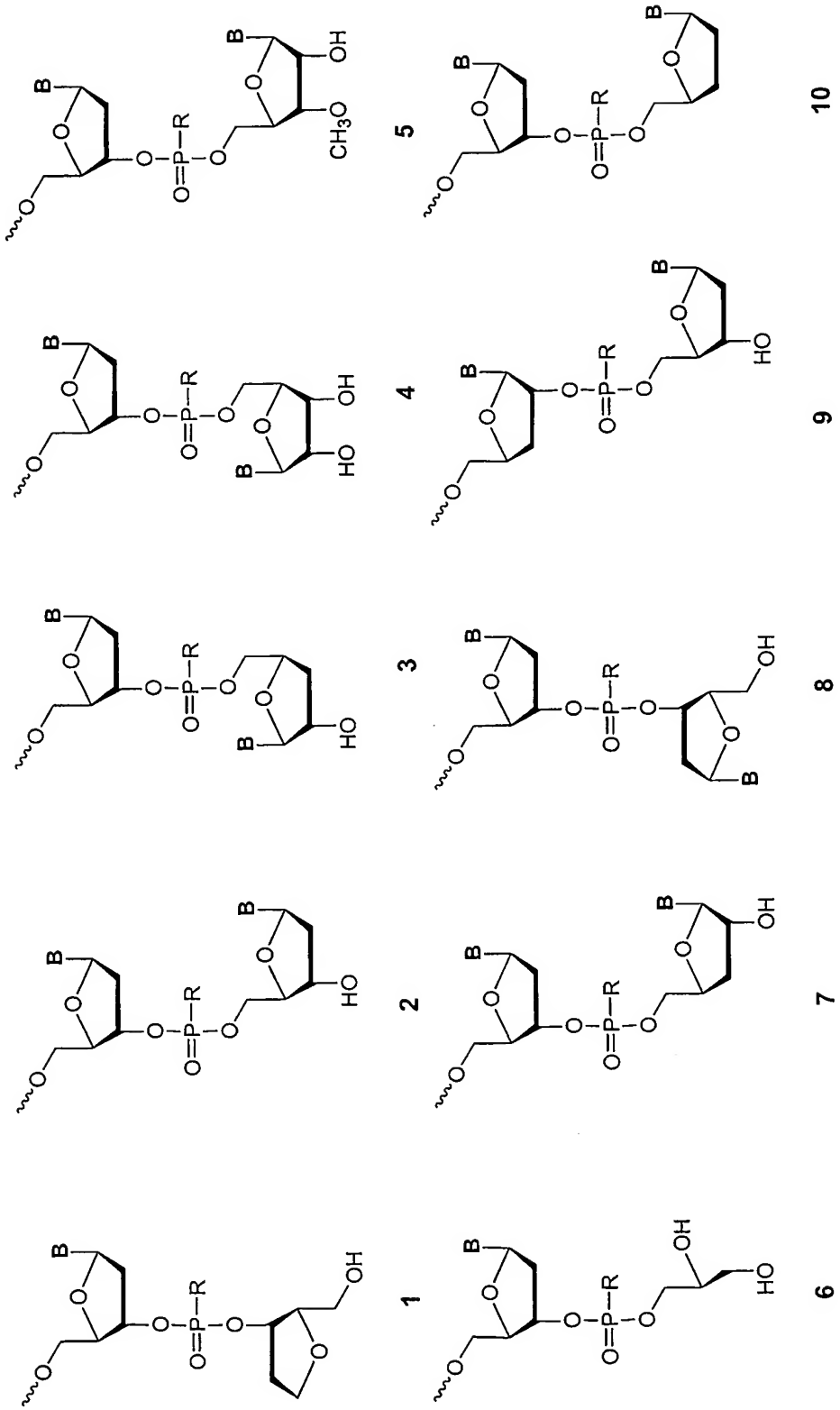


Figure 22



R = O, S, N, alkyl, substituted alkyl, O-alkyl, S-alkyl, alkaryl, or aralkyl
B = Independently any nucleotide base, either naturally occurring or chemically modified, or optionally H (abasic).

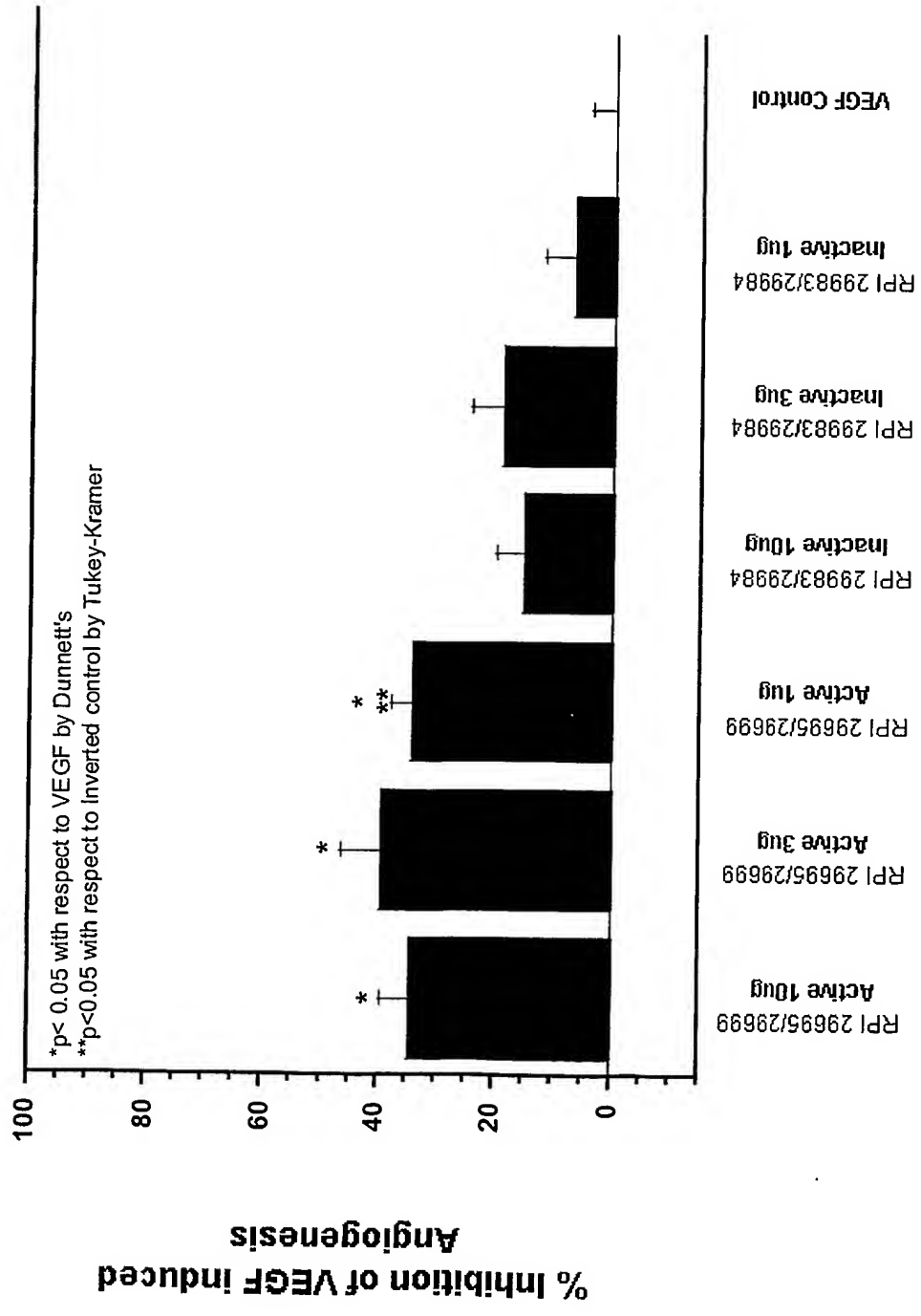


Figure 23: Inhibition of VEGF-Induced Angiogenesis by siRNAs

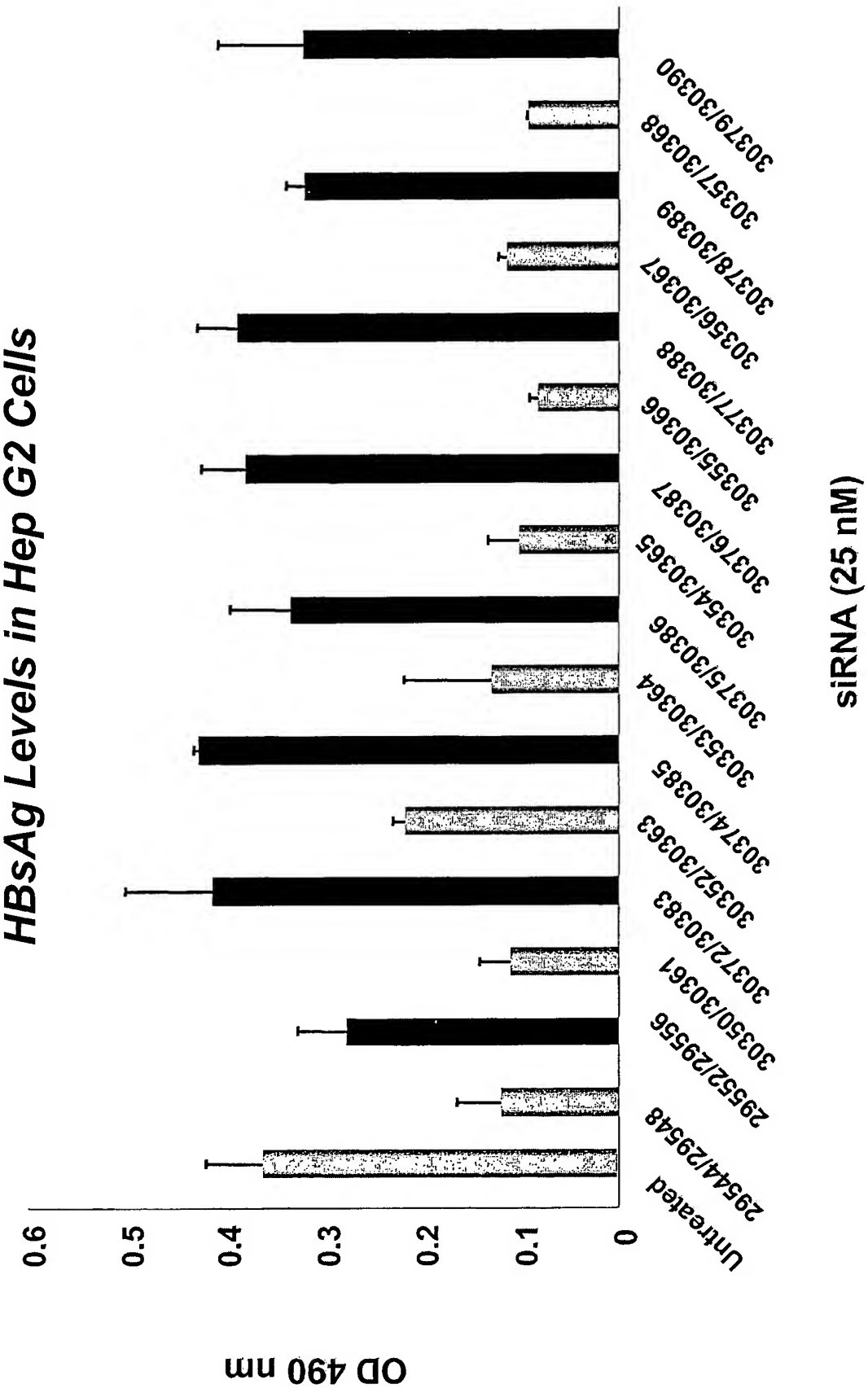
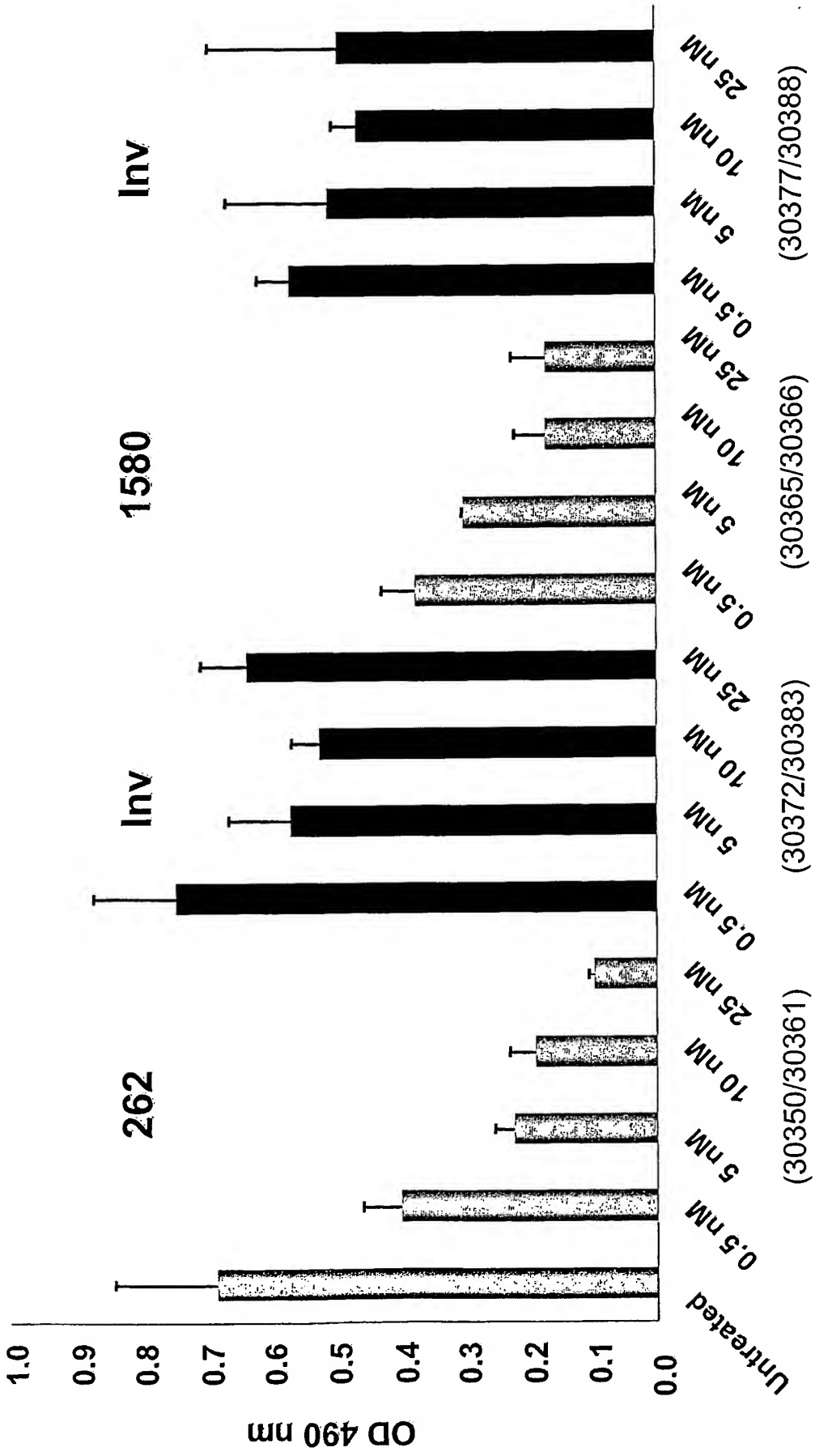


Figure 24: Stab4/5 siNA Targeted to HBV: HBsAg Levels in Hep G2 Cells

Figure 25: Dose Response with Stab4/5 siRNAs Targeted to HBV Sites 262 & 1580



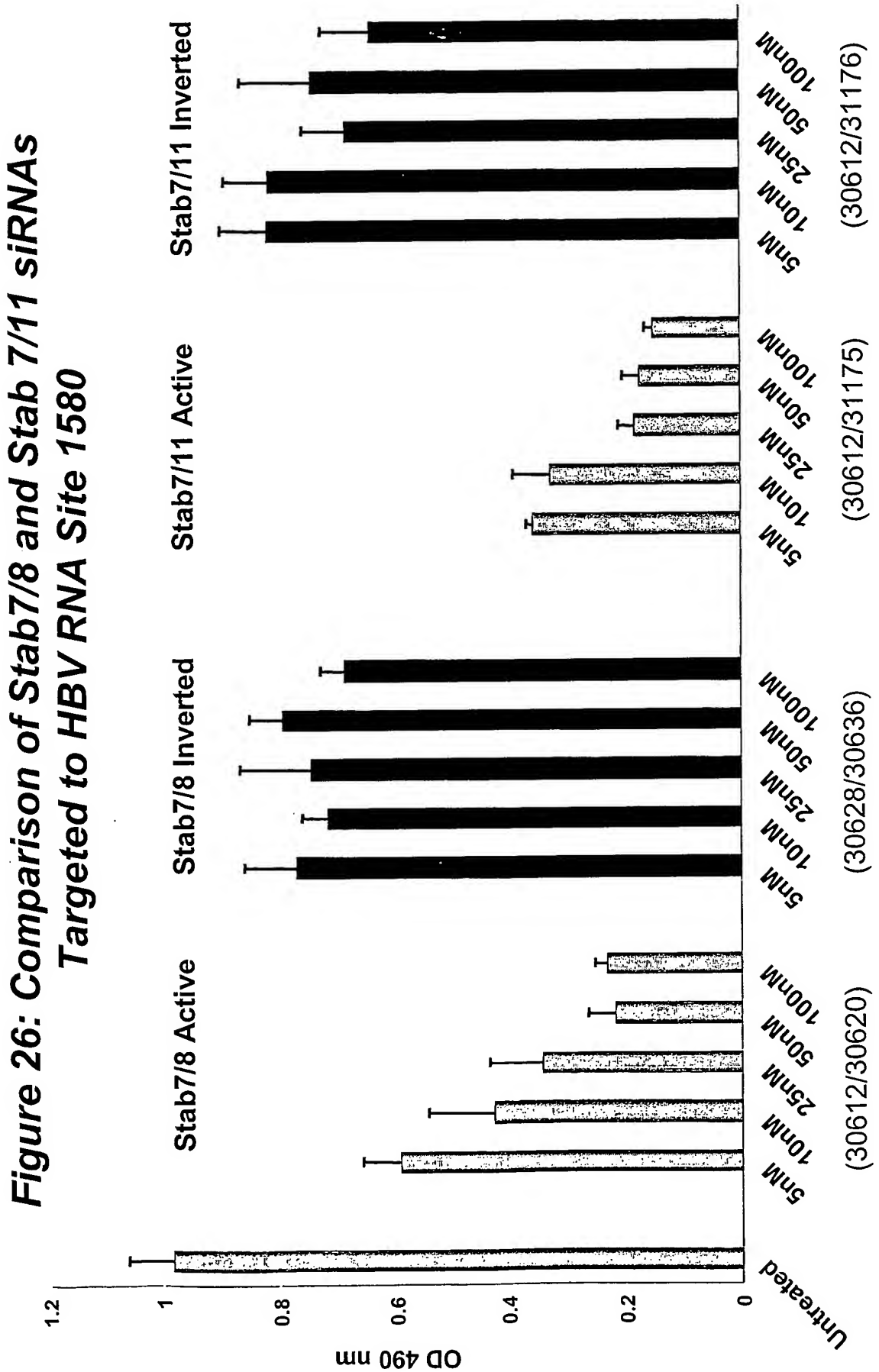
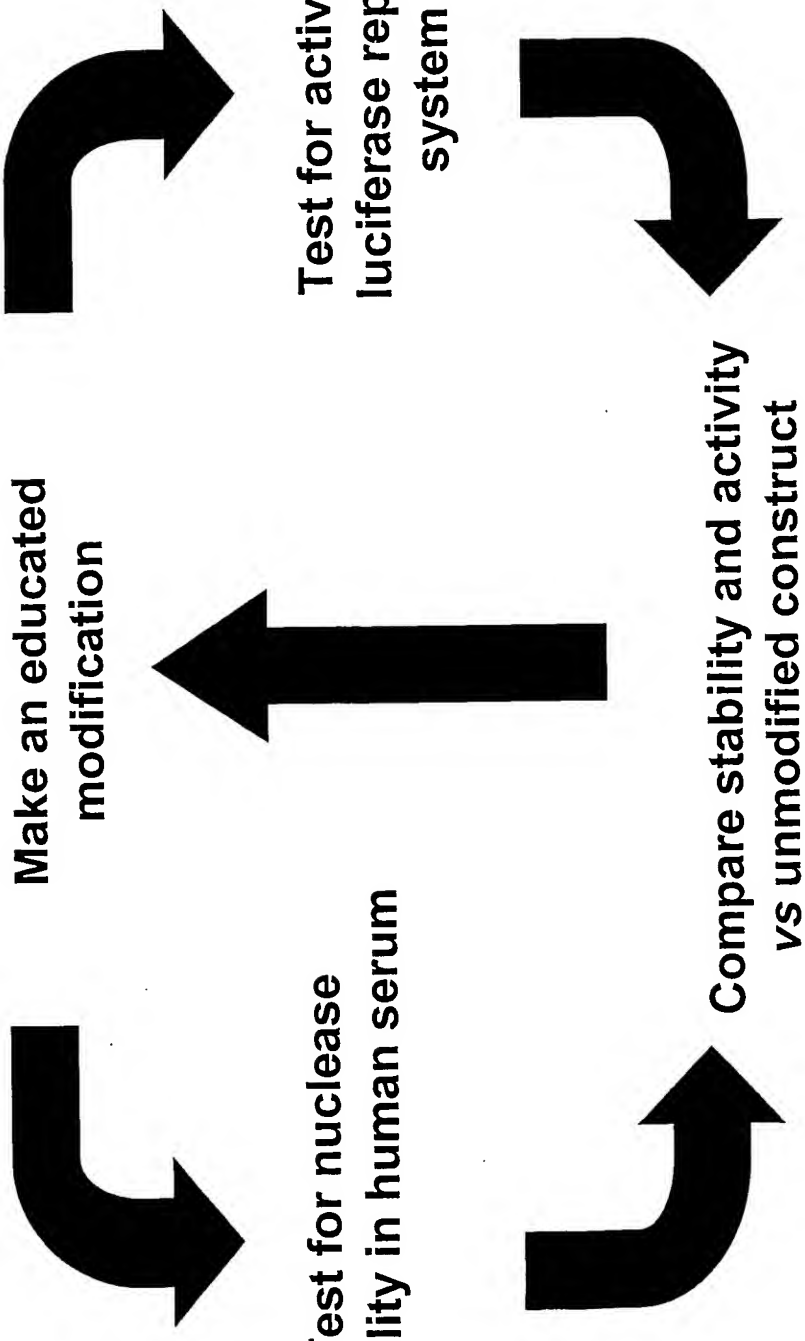


Figure 26: Comparison of Stab7/8 and Stab 7/11 siRNAs Targeted to HBV RNA Site 1580
OD 490 nm
1.2
1
0.8
0.6
0.4
0.2
0
Untreated
5nM
10nM
25nM
50nM
100nM
Stab7/8 Active
Stab7/8 Inverted
Stab7/11 Active
Stab7/11 Inverted
(30612/30620)
(30628/30636)
(30612/31175)
(30612/31176)

Figure 27: Modification Strategy



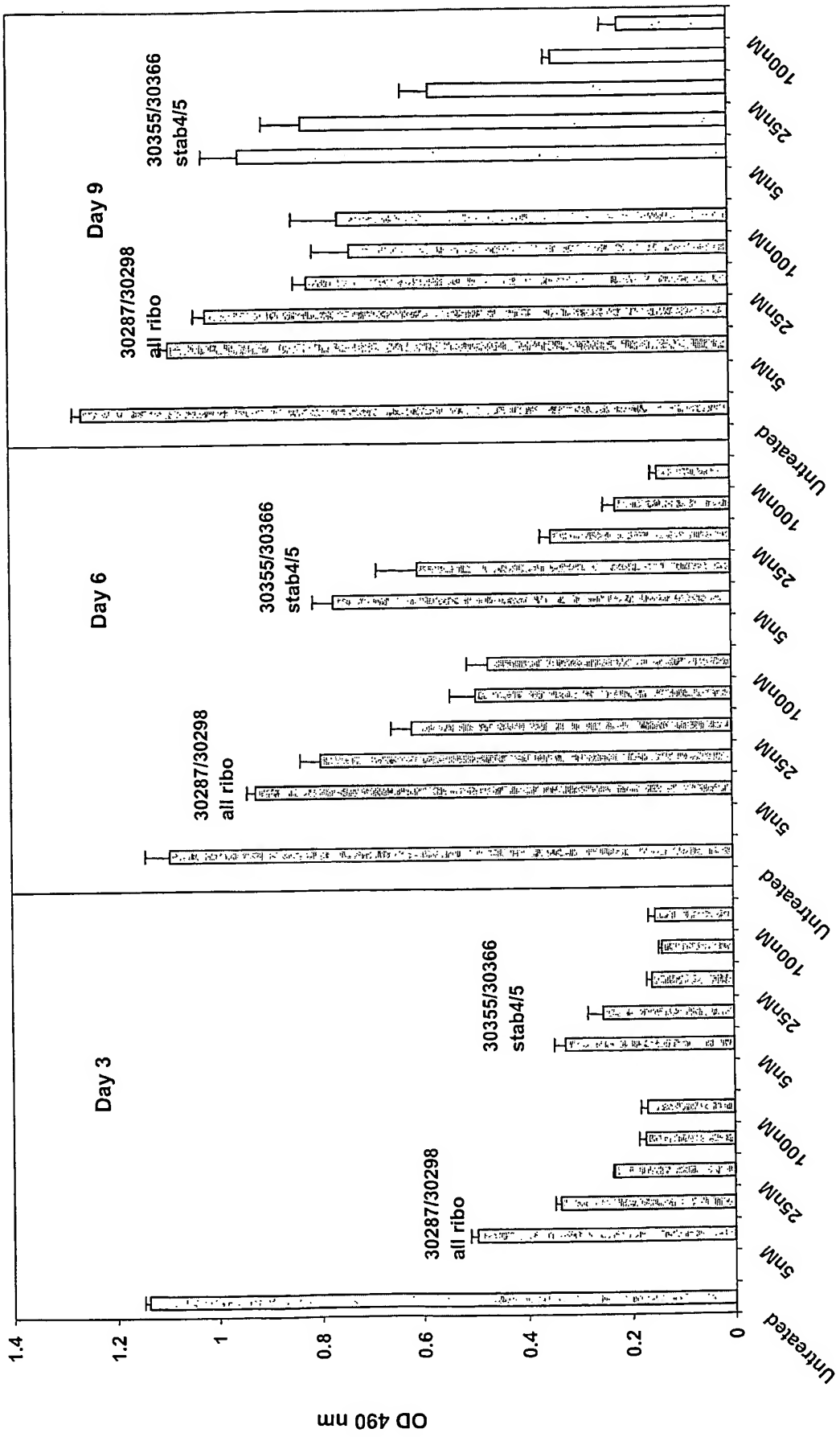


Figure 28: Duration of siRNA Effect
All-Ribo vs. Stab4/5 HBV Site 1580: HBsAg Levels

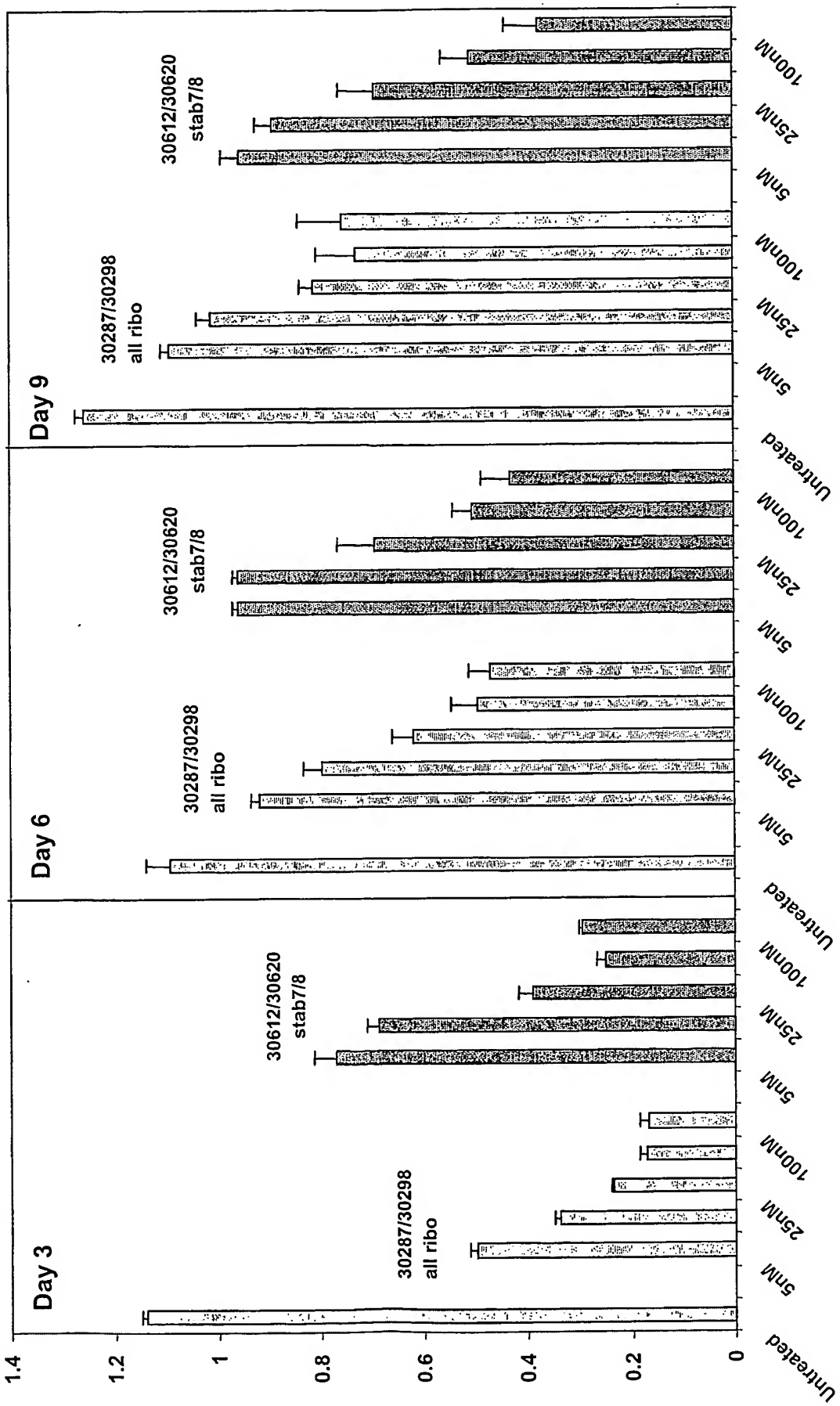


Figure 29: Duration of siRNA Effect All-Ribo vs. Stab7/8 HBV Site 1580: HBsAg Levels

Figure 30: Duration of siRNA Effect
All-Ribo vs. Stab7/11 HBV Site 1580: HBsAg Levels

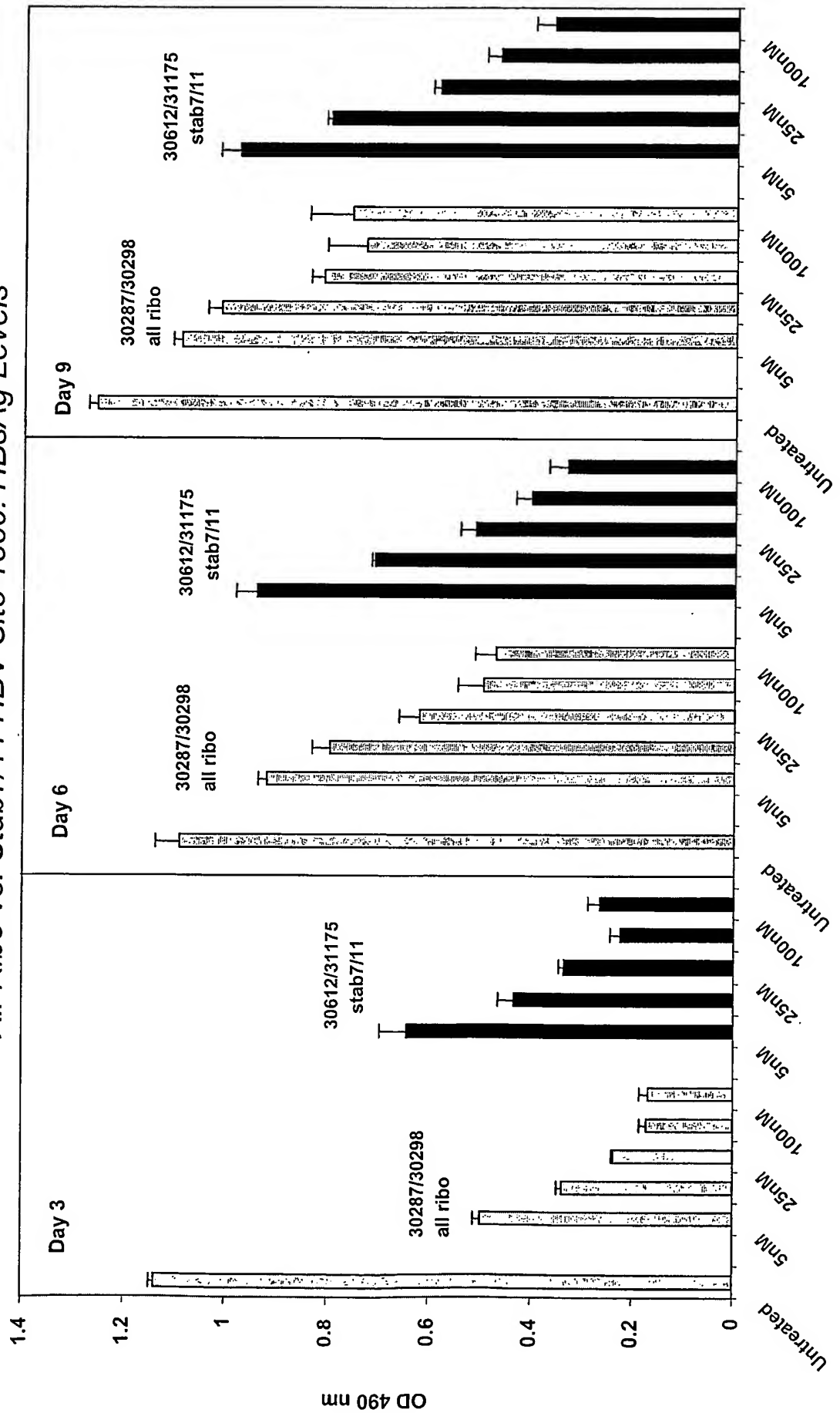


Figure 31: Duration of siRNA Effect
All-Ribo vs. Stab9/10 HBV Site 1580: HBsAg Levels

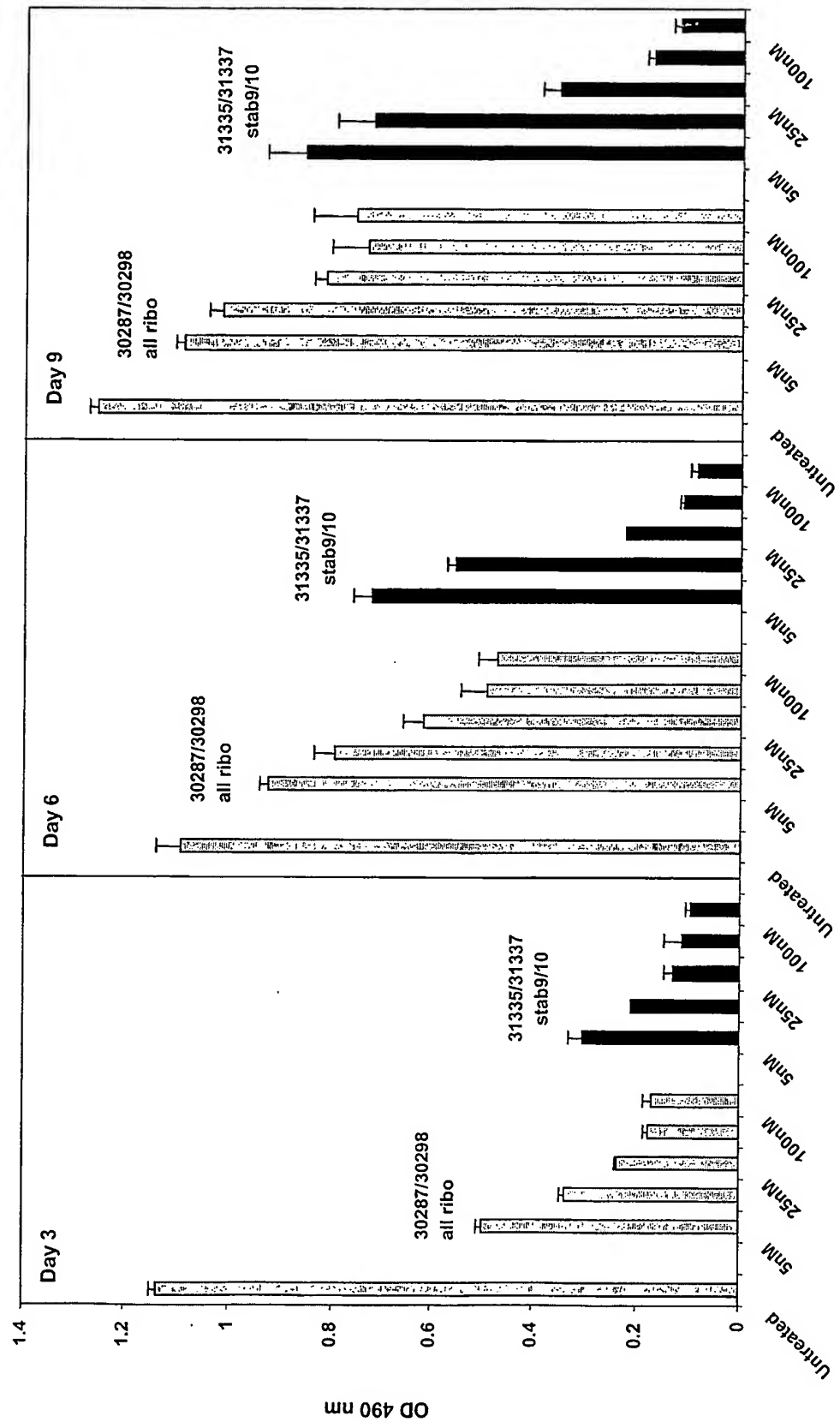


Figure 32 : siRNAs targeting HCV chimera

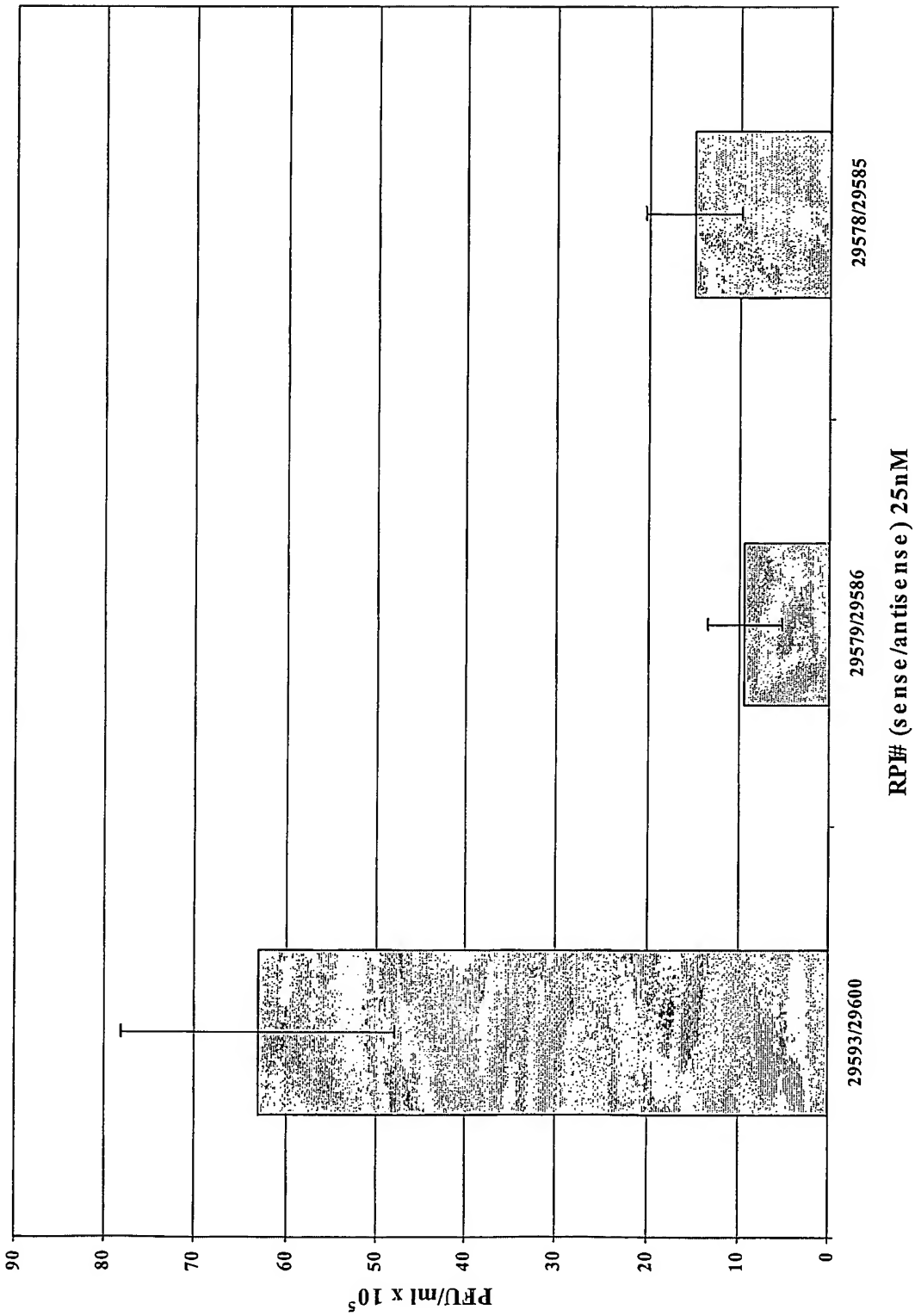


Figure 33: HCV siRNA dose response

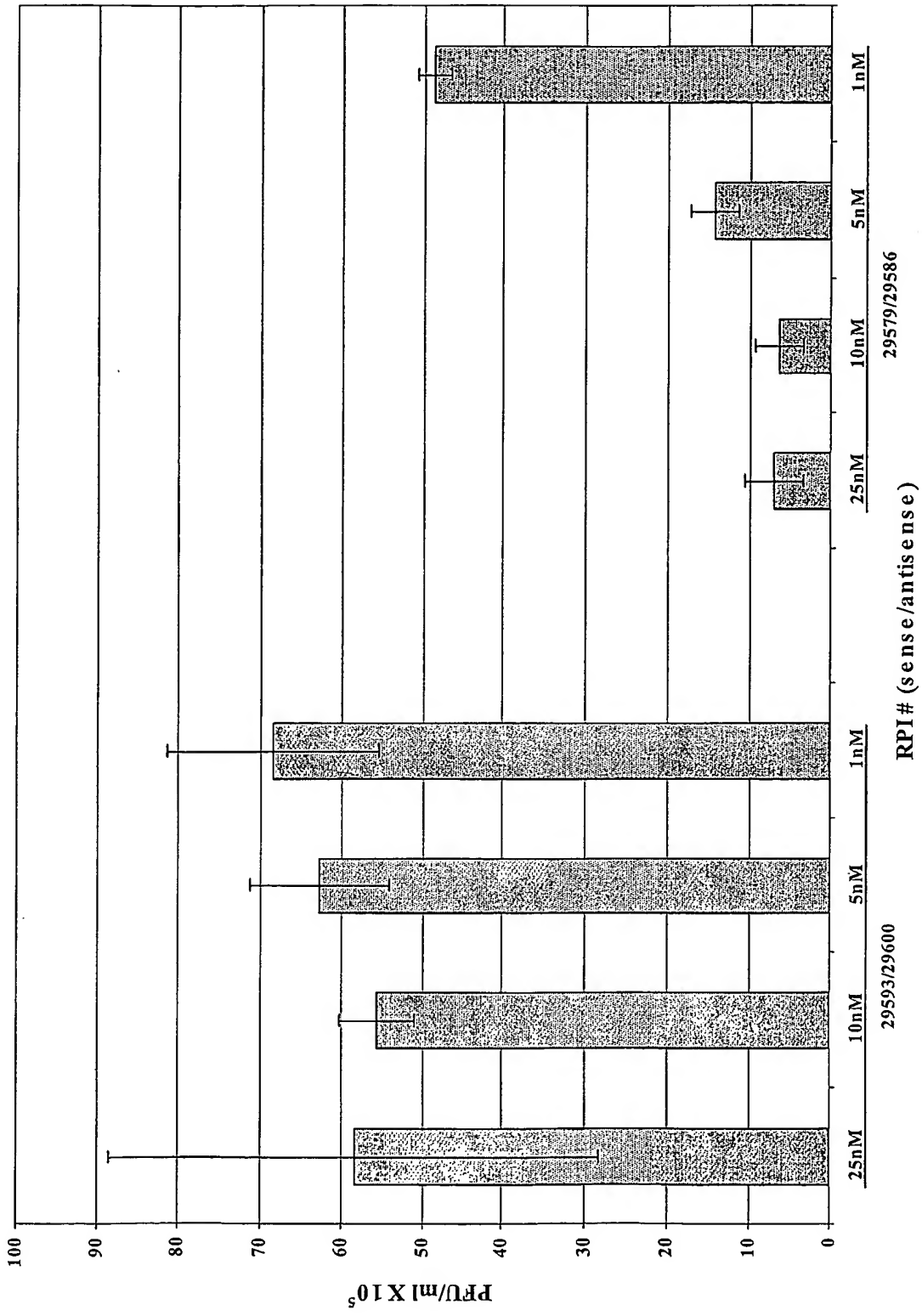


Figure 34: Chemically Modified siRNA targeting HCV chimera

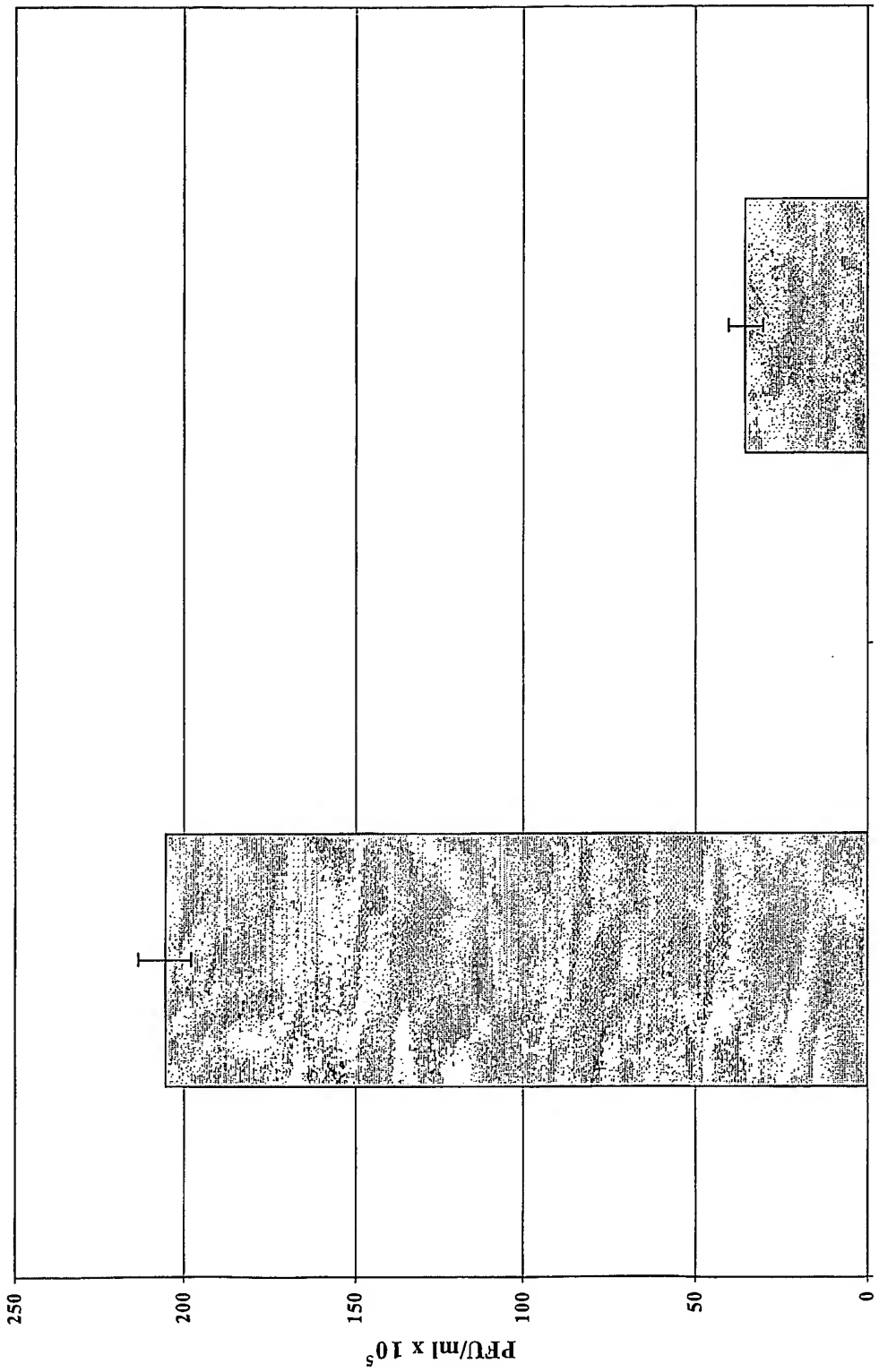


Figure 35: Chemically Modified siRNA targeting HCV chimera

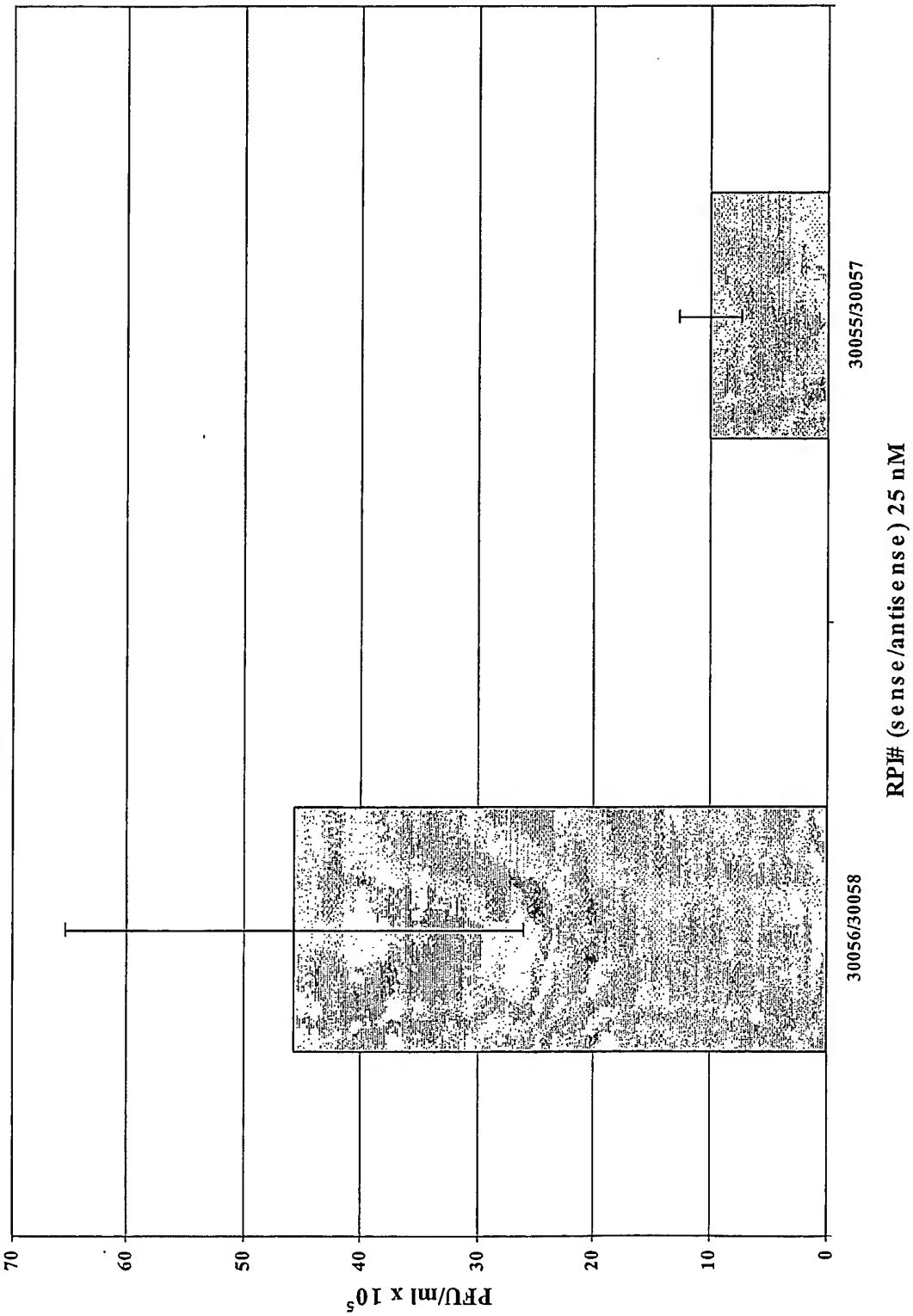


Figure 36: Chemically Modified siRNA targeting HCV chimera

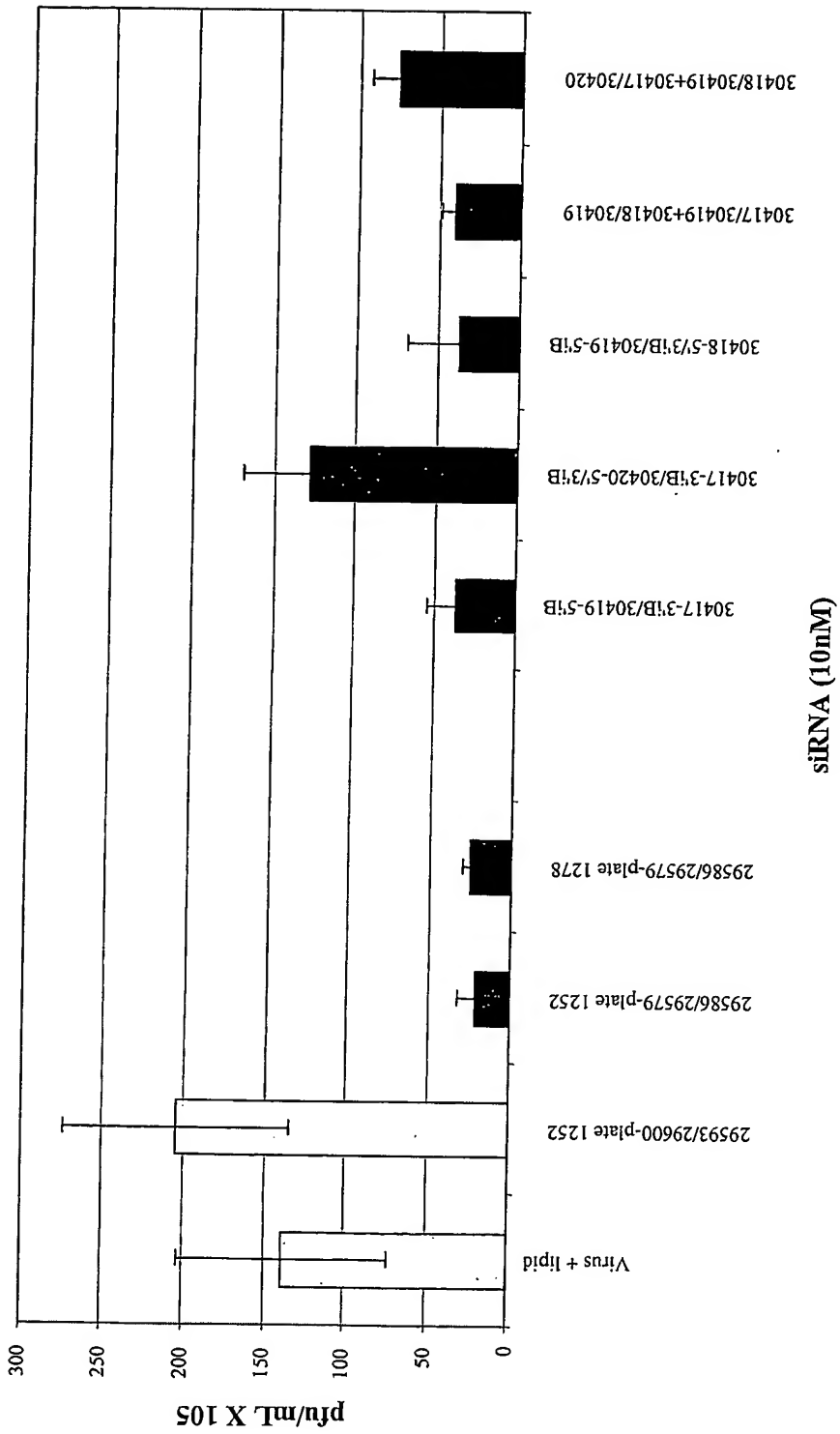
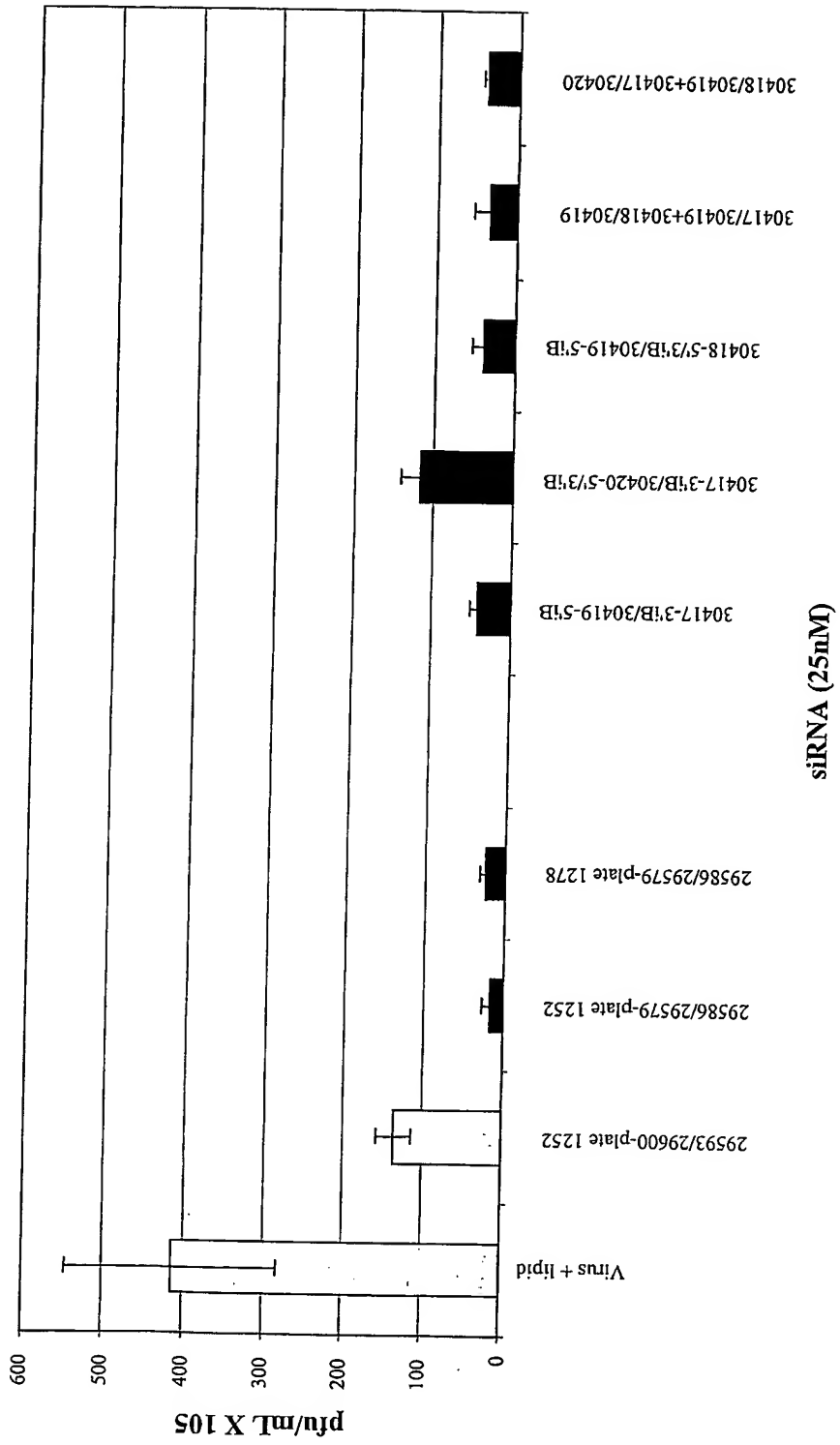


Figure 37: Chemically Modified siRNA targeting HCV chimera



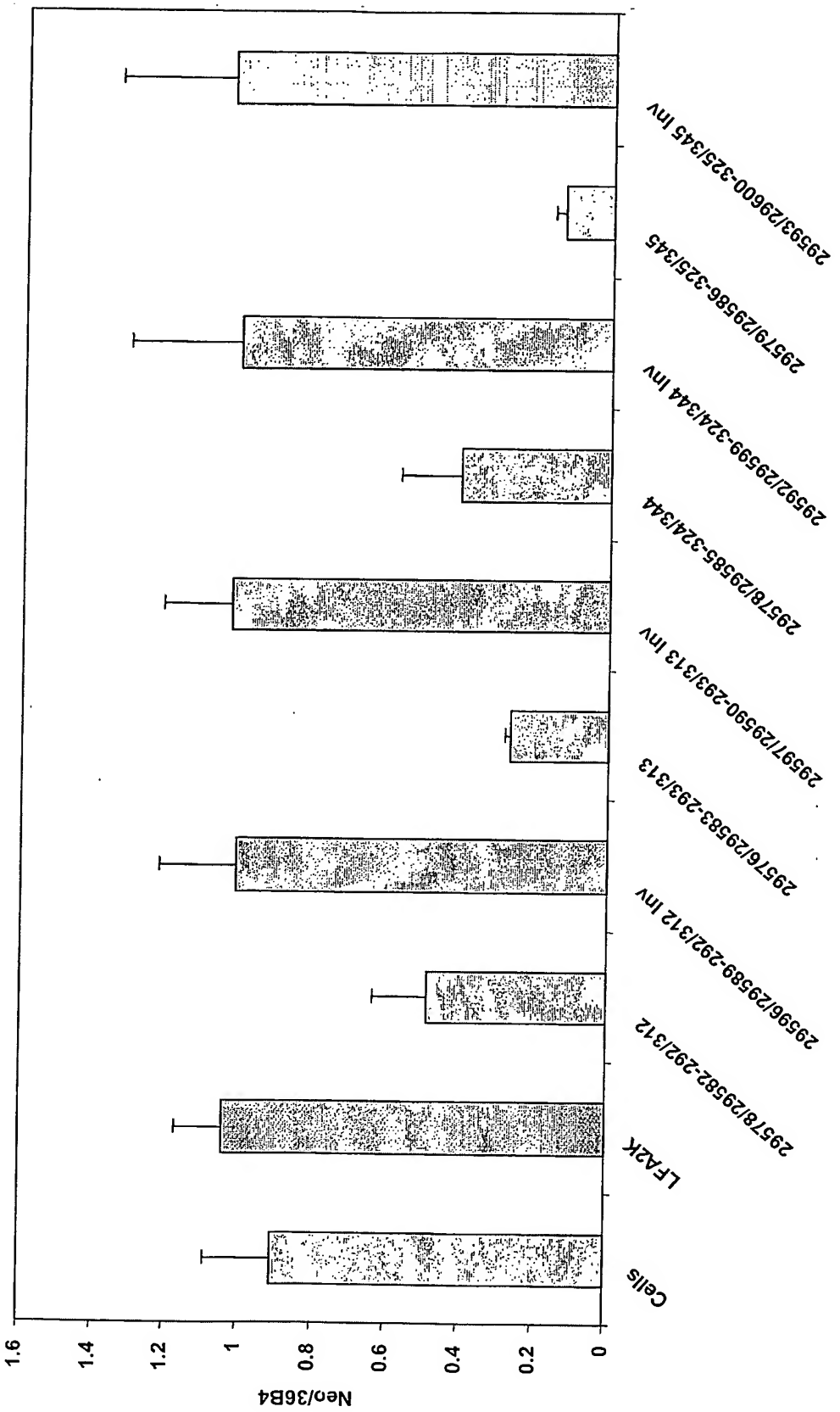


Figure 38: HCV/Replicon KJ#1-Clone A Cells transfected with 0.5µl/well LFA 2K-72 hours

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DOI: 10.1093/nar/gkg338

Similar behaviour of single-strand and double-strand siRNAs suggests they act through a common RNAi pathway

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ABSTRACT

RNA interference (RNAi), mediated by either long double-stranded RNA (dsRNA) or short interfering RNA (siRNA), has become a routine tool for transient knockdown of gene expression in a wide range of organisms. The antisense strand of the siRNA duplex (antisense siRNA) was recently shown to have substantial mRNA depleting activity of its own. Here, targeting human Tissue Factor mRNA in HaCaT cells, we perform a systematic comparison of the activity of antisense siRNA and double-strand siRNA, and find almost identical target position effects, appearance of mRNA cleavage fragments and tolerance for mutational and chemical backbone modifications. These observations, together with the demonstration that excess inactive double-strand siRNA blocks antisense siRNA activity, i.e. shows sequence-independent competition, indicate that the two types of effector molecules share the same RNAi pathway. Interestingly, both FITC-tagged and 3′-deoxy antisense siRNA display severely limited activity, despite having practically wild-type activity in a siRNA duplex. Finally, we find that maximum depletion of target mRNA expression occurs significantly faster with antisense siRNA than with double-strand siRNA, suggesting that the former enters the RNAi pathway at a later stage than double-strand siRNA, thereby requiring less time to exert its activity.

INTRODUCTION

In *Caenorhabditis elegans* specific depletion of mRNA was found both with the antisense and, surprisingly, the sense strand of RNA (1), leading to the discovery of RNA interference (RNAi) by Andrew Fire and co-workers (2). The potent RNAi process, whereby double-stranded (ds)RNA causes specific interference with the expression of the corresponding endogenous genes, appears to function in the defence against virus and transposons. This defence has subsequently been shown to exist in a wide range of species (3–5). With the demonstration of the efficacy of short interfering RNAs (siRNA) in human cells (6–8), a valuable tool for both research and therapeutics was created. Now the development has come full circle with the recent reports that ~21 nt single-stranded antisense siRNA is almost as potent as the siRNA duplex (9–11).

The double-strand siRNA appears to be incorporated into an inactive RISC complex, requiring unwinding of the duplex with concomitant loss of its sense strand for conversion into an active complex (RISC*) (12). The antisense siRNA is also incorporated into RISC in HeLa cell extracts and supports RISC-specific target RNA cleavage, although at lower efficiency than the siRNA duplex (9,10). The highly diverging estimates of the size of RISC (10,12,13), together with the reports of additional RISC-like complexes (14–17) associated with both siRNA and the related microRNAs (18), suggest the existence of various distinct complexes with possible involvement in different RNAi pathways.

Here we compare antisense and double-strand siRNA using a range of different oligos, demonstrating that the two types of effector molecules behave very similarly with regard to mRNA target position effects, cleavage fragment production and tolerance for chemical and mutational modifications. Furthermore, antisense siRNA can be blocked by competition with excess inactive double-strand siRNA. Interesting exceptions are antisense siRNAs modified in the 3′-terminus with either a 3′-deoxyribose instead of ribose or a FITC group in the 3′-OH position. These display severely limited activity, despite having practically wild-type activity in a siRNA duplex. Time-course experiments also showed a difference. They demonstrated that antisense siRNA depleted target mRNA faster than siRNA, possibly due to the direct incorporation of the former into RISC. Overall, our data suggest that antisense and double-strand siRNA work through the same RNAi pathway, although the 3′ end of the antisense strand may be treated differently in double-strand siRNA and antisense siRNA.

MATERIALS AND METHODS

RNAi antisense preparation

The 21 nt RNAs were synthesized as described (11,19). Antisense RNA were designated as-N, with N being the

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

Table 1. List of RNA molecules synthesized

Unmodified double-stranded and antisense siRNA	Strands/target position in human Tissue Factor
hTF167i	double-strand siRNA at hTF167
as-77	antisense siRNA at hTF77
as-158	antisense siRNA at hTF158
as-161	antisense siRNA at hTF161
as-164	antisense siRNA at hTF164
as-167	antisense siRNA at hTF167
as-170	antisense siRNA at hTF170
as-173	antisense siRNA at hTF173
as-176	antisense siRNA at hTF176
as-256	antisense siRNA at hTF256
as-372	antisense siRNA at hTF372
as-478	antisense siRNA at hTF478
as-562	antisense siRNA at hTF562
as-929	antisense siRNA at hTF929
Modified versions of antisense siRNA as-167	**Modification in antisense strand (nt, nucleotide, numbering from 5′ end)**
as-s3	mutated (C→G) at nt 17
as-s7	mutated (G→C) at nt 13
as-s10	mutated (C→G) at nt 10
as-s13	mutated (G→C) at nt 7
as-s16	mutated (G→C) at nt 4
as-M0+2	2-*O*-methylated 2 nt on 3′
as-M1+1	2-*O*-methylated 1 nt on 5′ and 3′
as-M2+2	2-*O*-methylated 2 nt on 5′ and 3′
as-M2+4	2-*O*-methylated 2 nt on 5′ and 4 nt on 3′
as-M4+4	2-*O*-methylated 4 nt on 5′ and 3′
as-M4+6	2-*O*-methylated 4 nt on 5′ and 6 nt on 3′
as-M6+6	2-*O*-methylated 6 nt on 5′ and 3′
as-M6+8	2-*O*-methylated 6 nt on 5′ and 8 nt on 3′
as-FITC	FITC-group on 3′ position on 3′ nt
as-3d	3′-terminal ribose replaced with 3′-deoxyribose
Modified versions of double-strand siRNA hTF167i	**Modifications in both strands (except hTF167-3d)**
M0+2	2-*O*-methylated 2 nt on 3′
M1+1	2-*O*-methylated 1 nt on 5′ and 3′
M2+2	2-*O*-methylated 2 nt on 5′ and 3′
M2+4	2-*O*-methylated 2 nt on 5′ and 4 nt on 3′
M4+4	2-*O*-methylated 4 nt on 5′ and 3′
M4+6	2-*O*-methylated 4 nt on 5′ and 6 nt on 3′
M6+6	2-*O*-methylated 6 nt on 5′ and 3′
M6+8	2-*O*-methylated 6 nt on 5′ and 8 nt on 3′
hTF167-3d	Antisense strand 3′-terminal ribose replaced with 3′-deoxyribose

corresponding siRNA and mRNA target position (19). The various mutated and chemically modified versions of as-167 were designated as-X, where X is the corresponding siRNA (11). A previously synthesized version of as-167 (as-3d) had a 3′-deoxyribose as the 3′-terminal nucleotide. The siRNAs mentioned in this paper are summarised in Table 1.

Cell culture and transfections

The human keratinocyte cell line HaCaT was cultured in serum-free keratinocyte medium (Gibco BRL) supplemented with 2.5 ng/ml epidermal growth factor and 25 µg/ml bovine pituitary extract. The cell line was regularly passaged at sub-confluence and plated 1 or 2 days before transfection. HaCaT cells in 6-well plates were transfected at low confluency (<40%) with 1.0 ml 100 nM double-strand siRNA in serum-free medium, using Lipofectamine 2000. For complexation, a 10 µM stock solution of double-strand siRNA or 20 µM stock solution of antisense siRNA was diluted with a 10× volume of serum-free medium and mixed with an equal volume of medium-diluted Lipofectamine 2000, at a ratio of liposome to siRNA of 5:2 v/w. Batch dilutions of liposomes were performed for each 6-well plate and preincubated at room temperature for 5–7 min before addition to the medium-diluted siRNA. Complexes were replaced with full medium 5 h after initiation of transfection. For standard assays of activity, cells were harvested the day after transfection. For longer incubations and time-course experiments, medium was replaced every second day after transfection. In time-courses with actinomycin D (Sigma), 10 µg/ml was added 2 h into the transfection period.

Northern analysis

Polyadenylated mRNA was isolated using Dynabeads oligo$(dT)_{25}$ (Dynal). Isolated mRNA was fractionated by electrophoresis for 16–18 h on 1.3% agarose/formaldehyde (0.8 M) gels and blotted onto nylon membranes

(MagnaCharge; Micron Separations). Membranes were hybridised with random primed Tissue Factor (position 61–1217 in cDNA) and GAPDH (1.2 kb) cDNA probes in PerfectHyb hybridisation buffer (Sigma).

RESULTS

Antisense siRNA shows lower activity than double-strand siRNA

We have previously shown that the maximum depletion of Tissue Factor mRNA by antisense siRNA, at 200 nM concentration, approaches that achieved by the corresponding siRNA at 100 nM (19). As double-strand siRNA-mediated inhibition reaches saturation at substantially lower concentrations than that used for antisense siRNA, the latter might still be substantially less active than double-strand siRNA at lower concentrations. Dose dependence experiments demonstrated that double-strand siRNA was active at ~5–6-fold lower concentrations than antisense siRNA (Fig. 1A). Their IC_{50} values were estimated at 5 and 30 nM, respectively.

Antisense siRNA has target position dependence

A comparison between antisense and double-strand siRNA cannot, of course, conclusively prove a shared RNAi pathway, but major deviations between the two could very well decisively disprove our hypothesis of a shared RNAi pathway. We therefore decided to investigate whether antisense siRNA would have the same position effects demonstrated previously for double-strand siRNA (19,20). The efficacies of antisense RNAs targeting sites in the Tissue Factor mRNA coding region from the start codon (as-77) to the stop codon (as-929) were evaluated in a quantitative northern assay (Fig. 1B). The results confirmed the position dependence of antisense siRNA efficacy, with the rank order of effectivity correlating strongly with inhibitory data for the corresponding double-strand siRNAs (hTF167 > hTF372 > hTF173, hTF164 > hTF256 > hTF161, hTF562) (Pearson's coefficient for bivariate correlation of inhibition 0.967, $P < 0.001$, $n = 7$).

Going from global position effects to investigate the local area on the mRNA close to the best antisense siRNA, as-167, using overlapping oligos differing only by 3 nt from each neighbour, we found that the activity differed sharply with each nucleotide triplet shift (Fig. 1C). The order of effectivity also correlated here with the double-strand siRNA efficacy order.

Antisense siRNA produces mRNA cleavage fragments

Evidence for RNAi-induced endonucleolytic cleavage of mRNA in mammalian cells was obtained in our previous work (19). Similar cleavage fragments were generated by the antisense siRNAs as-167 and as-372, targeting the two most accessible sites (Fig. 1B), with as-167 producing a larger cleavage fragment than as-372, as the as-372 target site is closer to the 3′ end on the target mRNA. This shows that antisense and double-strand siRNA both mediate endonucleolytic cleavage of target mRNA in a manner resulting in an apparent partial protection of a part of the degraded mRNA, possibly caused by kinetic or structural conditions. This strengthens the case for a common RNAi pathway.

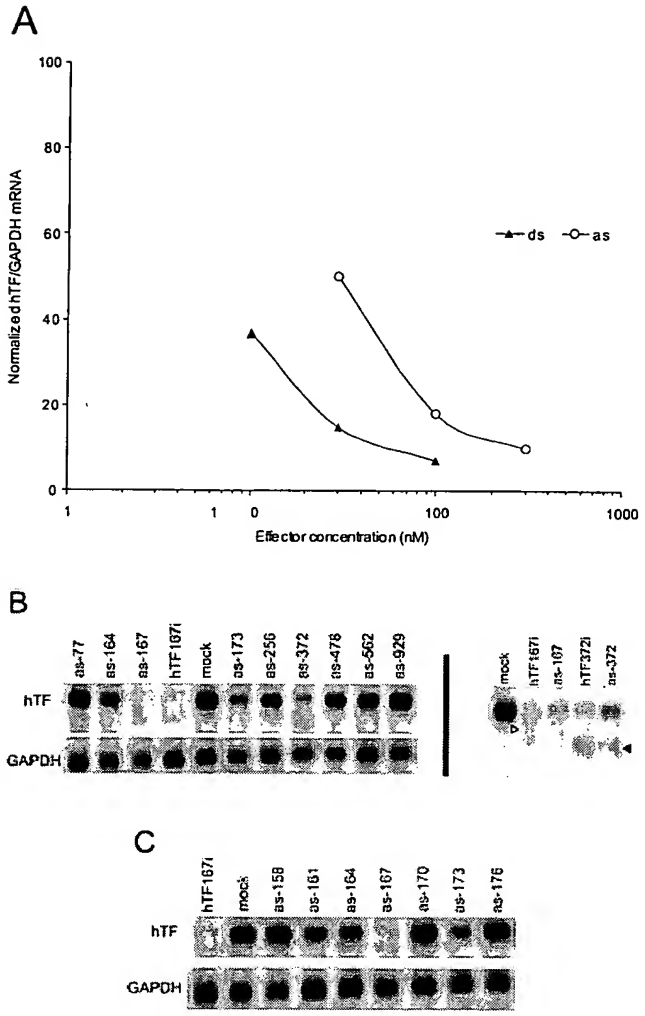

Figure 1. Antisense and double-strand siRNA show identical positional effects against the same target sites on Tissue Factor mRNA. (**A**) Dose dependence of double-strand (ds) and antisense (as) siRNA. Complexation with Lipofectamine 2000 was performed in one batch for all samples and complexes were diluted in medium immediately before addition to cells. (**B**) Global and (**C**) local target position effect. (B and C) Northern analysis of Tissue Factor mRNA after transfection of HaCaT cells with various antisense siRNA (200 nM) or double-strand (100 nM) siRNA. GAPDH was used as a control. Arrowheads (B, right panel) indicate cleavage products for antisense and double-strand siRNA.

Antisense siRNA is gradually affected by mutations

We have earlier demonstrated a relatively high tolerance for mutations in a double-strand siRNA with high activity (11). These mutated siRNAs, which display a wide range of activities, provided a further test of the correspondence between antisense and double-strand siRNA activity. Screening of a set of mutated antisense siRNAs demonstrated the same rank order of activity for the antisense candidates as for the corresponding siRNA duplexes (Fig. 2).

Antisense siRNA is gradually affected by nucleotide methylation

In a further characterisation of the functional anatomy of antisense siRNA, we investigated the effects on activity of

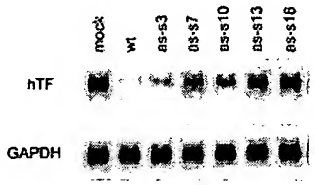

Figure 2. Mutational inactivation of the antisense siRNA as-167. Northern analysis of Tissue Factor mRNA after transfection of HaCaT cells with 200 nM as-167 (wild-type, wt) or single mutated (as-s3, as-s7, as-s10, as-s13 or as-s16; the numerals refer to the position of the mutation, counted from the 5′ end of the siRNA sense strand) versions of antisense siRNA as-167. GAPDH was used as a loading control.

gradually increasing degrees of 2′-*O*-methylation, as previously reported for double-strand siRNA (11). The mRNA depletion achieved with chemically modified antisense siRNA (Fig. 3A) mirrored the previously observed gradual attenuation of double-strand siRNA activity by chemical modification (11) (Fig. 3B). The antisense siRNA with very few modifications (as-M0+2 and as-M1+1) had almost the same activity as the wild-type as-167, while more extensive methylation resulted in a gradual decline in activity, more pronounced with antisense siRNA. Exceptions here are M2+2 and M2+4, which lost disproportionally more activity.

Antisense siRNA modified in the 3′-terminal 3′-OH position displays much lower activity than corresponding double-stranded siRNA

Another interesting difference in the effect of antisense and double-strand siRNA was seen with antisense siRNA tagged at the 3′ end with a fluorescent FITC group. This RNA oligo, when paired with the wild-type sense strand of the double-strand siRNA hTF167i, has previously been shown to have near wild-type activity (19). It was thus somewhat surprising that even at the highest achievable concentrations possible in our assays, the FITC-tagged antisense siRNA (as-FITC) demonstrated only very limited activity (data not shown). We first reasoned that the lack of activity was due to faulty incorporation into the RISC complex due to the bulky 3′ end fluorescent group. However, we find that an antisense siRNA with a 3′-deoxyribose modification (as-3d) of its 3′ end, although somewhat more active than the 3′-FITC version, has a substantially impaired activity compared with the wild-type antisense. In the corresponding siRNA duplex it has comparable activity with wild-type siRNA (Fig. 3C).

An excess of double-strand siRNA can competitively block antisense siRNA

The close similarity of antisense and double-strand siRNA mRNA depletion behaviour with regard to position effects, cleavage fragment formation, methylation and mutation tolerance suggest at least in part a common pathway for antisense and double-strand siRNA. If this is the case, the effect of antisense siRNA should be susceptible to competition by an inactive double-strand siRNA, in a manner previously shown for inactive and active siRNA (19,21). Competition experiments were therefore performed in order to test this hypothesis. These experiments are not straightforward to do, as discussed below. Nevertheless, a surplus of various inactive competitor double-strand siRNAs seemed to block the

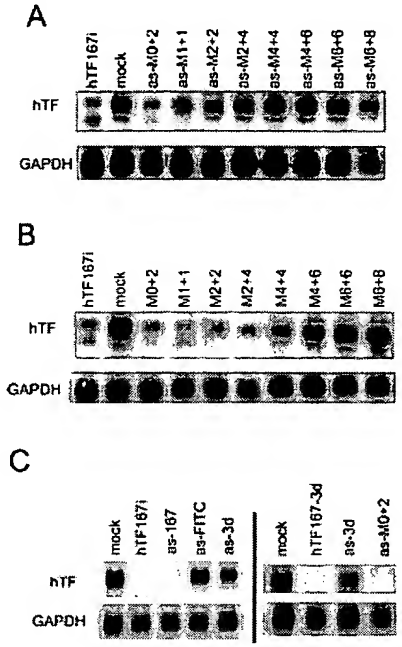

Figure 3. Comparison of the influence of chemical modification on antisense and double-strand siRNA. (**A**) Inactivation by methylation of antisense siRNA as-167. (**B**) Inactivation by methylation of double-strand siRNA hTF167i. (**C**) Inactivation of antisense siRNA by modification of the 3′-OH of the 3′-terminal nucleotide. as-FITC contains a FITC group in the 3′ position whereas in as-3d, 3′-deoxyribose is substituted for the terminal ribose. hTF167-3d is a double-strand siRNA in which as-3d is paired with a wild-type sense strand. (A–C) Northern analysis of Tissue Factor mRNA after transfection of HaCaT cells with 200 nM antisense siRNA or 100 nM double-strand siRNA. GAPDH was used as a loading control.

effect of the active antisense siRNA as-167 under various transfection conditions (Fig. 4).

Antisense siRNA reaches maximum activity faster than double-strand siRNA

Standard double-strand siRNA, in our Tissue Factor system, reaches maximum effect only after ~24 h, after which the silencing fades out over 3–5 days (19). The causes of the slow onset of silencing are not known. In our earlier work we proposed that RNAi depletion might be relatively slow and in a near equilibrium kinetic balance between mRNA depletion and mRNA transcription (19). Testing this concept by blocking mRNA transcription with actinomycin D showed that the natural turnover of mRNA, over a short period of 6 h, was faster than RNAi (Fig. 5A). This is consistent with the reported 1–2 h half-life of Tissue Factor mRNA (22,23). GAPDH has a reported half-life of >24 h (24,25), thus the 6 h exposure to actinomycin D should only reduce the GAPDH signal by <16%. Even when not considering decay of GAPDH, combining double-strand siRNA and actinomycin D gives an additive effect at all measured time points (Fig. 5A, lanes 4, 8 and 12). This might be interpreted as supporting the notion that RNAi has independent exonucleases for mRNA degradation (19,26,27).

Time-course experiments with antisense and double-strand siRNA demonstrated that the effect of the former was more transient, although effective silencing was still evident 3 days

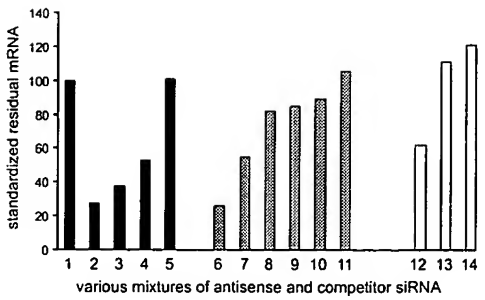

Figure 4. Competition between double-strand and antisense siRNA. Results in the black, grey and white columns are from three separate experiments. Experiments were performed in HaCaT cells as previously described, unless otherwise stated, and relative Tissue Factor/GAPDH expression normalised to levels in mock-transfected cells in each experiment. Column 1, mock; column 2, 50 nM antisense siRNA as-167; columns 3–5, 50 nM as-167 + 75 nM various inactive (column 3, hTF167-ds10/16) and irrelevant (column 4, PSK229i; column 5, BCR-ABL-1i) double-strand siRNA; column 6, 200 nM as-167; column 7, 50 nM as-167; columns 8–11, 50 nM as-167 + increasing concentrations (75, 100, 150 and 200 nM) of irrelevant double-strand siRNA (PSK208i); column 12, 200 nM as-167; column 13, 200 nM as-167 + 2000 nM PSK208i; column 14, 2000 nM PSK208i. In columns 12–14, cells were transfected with the less toxic agent Oligofectamine (Gibco BRL), enabling the use of higher total concentrations of RNA and excess of competitor, at the expense of reduced transfection efficiency.

post-transfection (Fig. 5B). In a further comparison of double-strand and antisense siRNA function, we have investigated the time dependence of the onset of mRNA depletion. Surprisingly, we found that the antisense siRNA effect was somewhat stronger than that of double-strand siRNA at 10 h post-transfection. The maximum effect of antisense siRNA seemed to be achieved already at this time point, as mRNA levels increased at later time points (Fig. 5C). This behaviour extended to the double-strand siRNA/antisense siRNA pair targeting hTF372 (data not shown). The faster onset of antisense siRNA-mediated silencing may be due to direct incorporation of single-stranded RNA into the active nuclease complex (RISC*) (12).

DISCUSSION

Antisense siRNA provides another efficient tool for use in transient knockdown of gene expression, but questions remain regarding its mechanism of function. This work has compared a range of different antisense siRNA oligos to their corresponding double-strand siRNA, seeking to disprove the hypothesis of a shared RNAi effector pathway by finding major differences between them in our different assays.

Antisense siRNA is somewhat less efficient than the corresponding double-strand siRNA, displaying an ~5-fold higher IC_{50}. The lower specific activity of antisense siRNA compared to double-strand siRNA may be due to a lower intracellular stability of the former. We expect antisense oligos to be protected by the transfecting agent until released from the lipid miscelles, a process that can take some time (19); following release from lipid miscelles and prior to incorporation into RISC, the antisense siRNA should be more vulnerable to degradation than double-strand siRNA. The above-mentioned drawbacks combined may result in a more transient nature of silencing, although our data suggest that antisense siRNA is able to maintain partial silencing for at least 3 days.

A standard set-up with 100 nM double-strand siRNA compared with 200 nM antisense siRNA was chosen to ensure equal amounts of both nucleic acids and the transfection agent. We found that the position dependence of antisense siRNA correlated with that of the corresponding double-strand siRNA. This observation has one important practical implication: it reduces the cost of screening for optimal target sites for siRNA. Despite its lower specific activity, antisense siRNA efficacy still approached that of the corresponding double-strand siRNA at moderate and non-toxic concentrations (200–300 nM).

When comparing antisense and double-strand with regard to both global and local mRNA target position effects, the appearance of cleavage fragments and the tolerance to mutational and chemical backbone modifications, a striking similarity of behaviour is evident. One would expect different effector complexes to have different target or oligo preferences, as previously reported for the different position dependencies of RNase H-competent ribozymes and siRNA (19,21). In the absence of significant differences between antisense and double-strand siRNA for a series of oligos, we propose that antisense and double-strand siRNA mediate RNAi through the same pathway, although we are not able to provide conclusive evidence for an identity of mechanism. An exception to the similarity of behaviour is as-FITC, a version of as-167 tagged with a bulky fluorescent FITC group at the 3′-OH position of the 3′-terminal nucleotide, which displayed very poor activity. We first interpreted these results to indicate the impairment of incorporation of the antisense strand into the active RISC* due to the presence of a bulky functional group. However, a version with 3′-deoxyribose instead of ribose at the 3′-terminus of the antisense siRNA, as-3d, showed similar behaviour. Efforts to investigate this phenomenon further are currently under way.

Direct proof of a shared RNAi pathway *in vivo* would be the demonstration of inhibition by competition. Unfortunately, this proof is hard to come by as lipid toxicity sets an upper limit for oligo concentrations and antisense siRNA requires higher doses than double-strand siRNA. Furthermore, difficulties arise from the surplus capacity of the RNAi system under optimal conditions, where blocking part of the excess capacity, for example by lowering of the oligo concentration or partial inactivation of oligos by chemical modification or mutation, does not affect the final mRNA depletion activity (11,19). Finally, the entry of antisense siRNA at a supposedly later point in the pathway leaves antisense siRNA alone in the RISC* for a limited period, setting further limitations on a competition assay. This last point has been emphasised by the recent *in vitro* demonstration that competition for formation of a target-specific RISC required the simultaneous addition of both active and competitor siRNA to the lysate; competitor added 15 min after initiation of incubation could no longer compete with the active siRNA (10). The relevance of this observation for a biologically necessary turnover *in vivo* over hours and days is doubtful. Still, with the caveats noted, it has proved possible to block antisense siRNA with inactive or irrelevant double-strand siRNA, thus strengthening the hypothesis of a shared pathway.

Finally, we demonstrate that natural turnover of Tissue Factor mRNA is faster than RNAi, and that the two effects are additive. This can be interpreted as support for the existence of

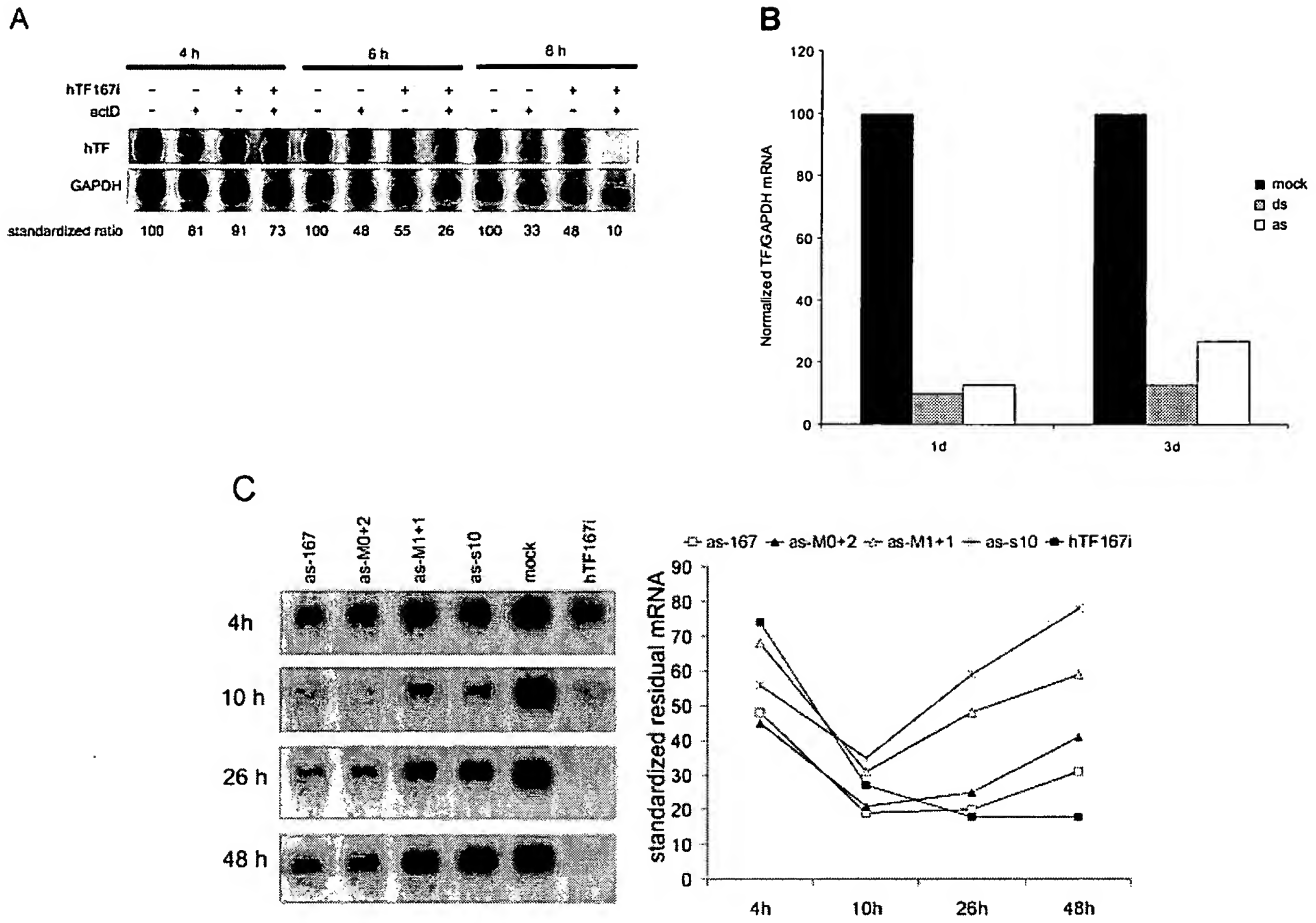

Figure 5. Time-courses of the efficacy of double-strand and antisense siRNA. (**A**) Actinomycin D time-course. Cells were transfected with double-strand siRNA (100 nM) hTF167i or mock control as indicated above each lane. Actinomycin D (10 μg/ml) was added to the medium 2 h later and cells harvested at 4, 6 and 8 h post-transfection as indicated. The Tissue Factor signal was standardised to GAPDH and normalized to levels in the control at each time point. The results are representative for two independent experiments. (**B**) Long-term effect of antisense (as) and double-strand (ds) siRNA targeting hTF167. Tissue Factor mRNA was measured at days 1 and 3 post-transfection and standardized to a GAPDH control. Data are from one of two independent experiments. (**C**) Short-term time-course of antisense and double-strand siRNA mRNA depletion. Cells were transfected as described and harvested at the indicated time points. Tissue Factor/GAPDH expression was normalised to levels in mock-transfected cells. The results are representative of two independent experiments.

independent RNAi exonucleases (19,26,27). Interestingly, the slow onset of double-strand siRNA-mediated was contrasted by the early onset of antisense siRNA-mediated mRNA depletion. This argues that the antisense siRNA enters the RNAi pathway at a stage closer to the effector nuclease than double-strand siRNA. The unwinding of the RNA strands, perhaps mediated by one of the putative helicases recently found to be associated with RISC and RISC-like complexes (14,17), could thus constitute a rate-limiting step in the pathway, partially explaining the slow onset of double-strand siRNA-mediated silencing (19). Furthermore, this discovery of differential depletion speed could give rise to two different classes of therapeutic drugs if RNAi finds clinical application.

ACKNOWLEDGEMENTS

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/019229

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/11 A61K31/7088 A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	US 6 518 417 B1 (SCZAKIEL GEORG AND PATZEL VOLKER) 11 February 2003 (2003-02-11) the whole document column 2, lines 15-22; sequences 4-121 column 4, lines 51-56 -----	21,24, 25,27, 30,31 1-4, 13-15,20
Y	HOLEN T ET AL: "Similar behaviour of single-strand and double-strand siRNAs suggests they act through a common RNAi pathway" NUCLEIC ACIDS RESEARCH, OXFORD UNIVERSITY PRESS, SURREY, GB, vol. 31, no. 9, 1 May 2003 (2003-05-01), pages 2401-2407, XP002281439 ISSN: 0305-1048 the whole document -----	1-4, 13-15,20

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
17 December 2004	16. 09. 2005

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Macchia, G

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/019229

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	GILADI H. ET AL.: "Small interfering RNA inhibits hepatitis B virus replication in mice" MOLECULAR THERAPY, vol. 8, no. 5, November 2003 (2003-11), pages 769-776, XP008040415 siRNA-1 the whole document	1,2,13, 21
P,X	WO 03/070918 A (RIBOZYME PHARMA INC (US) MCSWIGGEN BEIGELMAN MACEJAK ZINNEN PAVCO ET A) 28 August 2003 (2003-08-28) page 75, lines 15-21 SEQ ID NO:425, 426: RPI#30350/30361 page 146 page 116 - page 118; figures 24,25; example 13	1-4, 13-15, 20,21, 24,30,31
A	MCCAFFREY A.P. ET AL.: "Inhibition of hepatitis B virus in mice by RNA interference" NATURE BIOTECHNOLOGY, vol. 21, no. 6, 1 June 2003 (2003-06-01), pages 639-644, XP008040439 HBVU6no.1 the whole document	
A	ANDINO R.: "RNAi puts a lid on virus replication" NATURE BIOTECHNOLOGY, vol. 21, no. 6, 1 June 2003 (2003-06-01), pages 629-630, XP002311173 the whole document	
A	SHLOMAI A. AND SHAUL Y.: "Inhibition of hepatitis B virus expression and replication by RNA interference" HEPATOLOGY, vol. 37, no. 4, April 2003 (2003-04), pages 764-770, XP008040408 the whole document	
A	COUZIN J.: "Mini RNA molecules shield mouse liver from hepatitis" SCIENCE, vol. 299, 14 February 2003 (2003-02-14), page 995, XP002310940 the whole document	

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/019229

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	HAMASAKI K. ET AL.: "Short interfering RNA-directed inhibition of hepatitis B virus replication" FEBS LETTERS, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL, vol. 543, no. 1-3, 22 May 2003 (2003-05-22), pages 51-54, XP004425032 ISSN: 0014-5793 the whole document	
A	WILSON J.A. ET AL.: "RNA interference blocks gene expression and RNA synthesis from hepatitis C replicons propagated in human liver cells" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 100, no. 5, 4 March 2003 (2003-03-04), pages 2783-2788, XP002300963 ISSN: 0027-8424 the whole document	
A	MCCAFFREY A.P. ET AL.: "RNA interference in adult mice" NATURE, MACMILLAN JOURNALS LTD. LONDON, GB, vol. 418, no. 6893, 4 July 2002 (2002-07-04), pages 38-39, XP002234152 ISSN: 0028-0836 the whole document	
A	KAPADIA S.B. ET AL.: "Interference of hepatitis C virus RNA replication by short interfering RNAs" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 100, no. 4, 18 February 2003 (2003-02-18), pages 2014-2018, XP002251050 ISSN: 0027-8424 the whole document	
A	SEO MI YOUNG ET AL.: "Small interfering RNA-mediated inhibition of hepatitis C virus replication in the human hepatoma cell line Huh-7" JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 77, no. 1, January 2003 (2003-01), pages 810-812, XP002261781 ISSN: 0022-538X the whole document	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2004/019229

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-14 and 9-13 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [X] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-4, 9-15, 18-21, 23-27, 30, 31 all partially

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-4, 9-15, 18-21, 23-27, 30, 31 all partially

A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus by means of a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:1.
Methods, compositions, polynucleotide sequences, expression constructs and mammalian cells related thereto.
A polynucleotide sequence comprising at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:25 or SEQ ID NO:26.

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2. claims: 1-4, 9-15, 18-21, 23-27, 30, 31 all partially

A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus by means of a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:2.
Methods, compositions, polynucleotide sequences, expression constructs and mammalian cells related thereto.
A polynucleotide sequence comprising at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:23 or SEQ ID NO:24.

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3. claims: 1-4, 9-15, 18-21, 23-27, 30, 31 all partially

A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus by means of a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:3.
Methods, compositions, polynucleotide sequences, expression constructs and mammalian cells related thereto.
A polynucleotide sequence comprising at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21 or SEQ ID NO:22.

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4. claims: 1-4, 9-15, 18-21, 23-27, 30, 31 all partially

A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus by means of a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:4.
Methods, compositions, polynucleotide sequences, expression constructs and mammalian cells related thereto.
A polynucleotide sequence comprising at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:16 or SEQ ID NO:17.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

5. claims: 1-4, 9-15, 18-21, 23-27, 30, 31 all partially

A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus by means of a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8.
Methods, compositions, polynucleotide sequences, expression constructs and mammalian cells related thereto.
A polynucleotide sequence comprising at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:14 or SEQ ID NO:15.

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6. claims: 1-4, 9-15, 18-21, 23-25, 30, 31 all partially

A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus by means of a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:9.
Methods, compositions, polynucleotide sequences, expression constructs and mammalian cells related thereto.

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7. claims: 1-4, 9-15, 18-21, 23-27, 30, 31 all partially

A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus by means of a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:10.
Methods, compositions, polynucleotide sequences, expression constructs and mammalian cells related thereto.
A polynucleotide sequence comprising at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:18.

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8. claims: 5-13, 16-20, 22-25, 30, 31 all partially

A method for inhibiting expression of a polynucleotide sequence of hepatitis C virus by means of a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:11.
Methods, compositions, polynucleotide sequences, expression constructs and mammalian cells related thereto.

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9. claims: 5-13, 16-20, 22-25, 30, 31 all partially

A method for inhibiting expression of a polynucleotide sequence of hepatitis B virus by means of a double-stranded RNA effector molecule comprising an at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:12.
Methods, compositions, polynucleotide sequences, expression constructs and mammalian cells related thereto.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

10. claims: 28-31 all partially

A polynucleotide sequence comprising at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:27.
Expression construct and mammalian cell related thereto.
A polynucleotide sequence comprising at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:33, SEQ ID NO:34, SEQ ID NO:35, SEQ ID NO:36, SEQ ID NO:37, SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43 or SEQ ID NO:44.

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11. claim: 28 partially

A polynucleotide sequence comprising at least 19 contiguous base pair nucleotide sequence from SEQ ID NO:28.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US2004/019229

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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